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on Chromosome 18q21

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The majority of breast cancer cases is of sporadic origin and is usually diagnosed at an advanced stage. Mutations in the <i>Smad2</i> and <i>Smad4</i> genes, localized to chromosome 18q, a region frequently deleted in advanced cancers is rare in breast cancer unlike pancreatic, colon, lung and ovarian cancers. These findings support the existence of other tissue specific genes that are specifically targeted for inactivation in breast cancer. Our survey of the various <i>Smad</i> genes using a novel technique known as "TEGD" (targeted expressed gene display) has provided the first clues in identifying the <i>Smad8</i> gene as an important target for loss of expression in nearly 30% of breast cancers, a level of alteration similar to that of the <i>HER/neu</i> gene, a celebrated tumor marker for breast cancer. These studies further suggest that loss of expression of the <i>Smad4</i> gene or over-expression of the <i>Smad7</i> gene could be important in breast cancer. Our data is consistent with the hypothesis that disabling the Smad signaling pathway could be an important step in the genesis of breast cancer. Further studies to unravel the other modes of alterations of <i>Smad</i> genes as well as alternate targets in the Smad signaling pathways are in progress.				
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1. Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGF- β and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.
2. Thiagalingam, S., R. L. Foy, K-h.Cheng, H. J. Lee, A. Thiagalingam, and J. F. Ponte. 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. *Current Opinion in Oncology* 14(1): 65-72.

Appendix II-Resume: Dr. Sam Thiagalingam

ANNUAL REPORT OF THE USAMRMC FUNDED ACTIVITY

Title of the grant: Metastatic progression of breast cancer by allelic loss on chromosome 18q21.

1. Introduction/ Project Overview/ Scientific Progress and future directions:

The majority of molecular genetic studies on breast cancer have focused on familial predisposition and there has been a lack of serious effort to understand the molecular basis of the involvement of genetic determinants in the progression to metastatic cancer. The fact that 18q loss has been associated with the advanced carcinoma stage of other cancers suggests that the genes inactivated by this alteration in breast cancer could also be potentially associated with the conversion of benign tumors to malignancy and metastatic progression of the cancer. Unlike pancreatic, colon, lung and ovarian cancers, the lack of mutations in breast cancer in the *Smad2* and *Smad4* genes, localized to chromosome 18q supports the existence of tissue specific genes that are specifically targeted for inactivation in breast cancer and the urgent need for their identification.

Disabling Smad signaling in cancer has become increasingly recognized as an important step that affects processes such as loss of growth inhibition, promotion of angiogenesis and metastasis and the epithelial mesenchymal transition (1). Although significant progress has been made in elucidating the association between genetic alterations in the *Smad4* gene and cancer, the nature of defects involving the other Smads has been elusive, potentially due to alternative mechanisms or targets that result in the loss of or altered signaling end effects. Our survey of the various *Smad* genes has provided the first clues in identifying the *Smad8* gene as an important target for loss of expression in nearly 30% of breast cancers. The epigenetic alterations that underlie these overall abnormalities in signaling could occur at the level of regulation of gene expression or processing of the transcripts. We believe that it is a significant finding as even the most celebrated tumor marker, *HER/neu* gene amplification, also occurs in about 20%-30% breast cancer cases. We hypothesize that *Smad* signaling downstream of the BMPs involving *Smad8* could be an important pathway in breast tissue and inactivation or loss of *Smad8* is a critical tissue specific event in breast tumorigenesis.

On the other hand, there is also emerging data, which supports a potential role for signaling events mediated by the *Smad4* gene, localized to 18q21, in the metastatic progression of cancer. Recent studies suggest that disabling *Smad4* signaling leads to increased expression of VEGF (vascular endothelial growth factor), a primary regulator of vascular development, which plays a critical role in angiogenesis and metastasis, and decreased expression of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis (2). We therefore hypothesize that normal *Smad4* signaling is required to maintain suppression of metastasis, and inactivation of this signaling is a major step in the development of metastatic breast cancer. Chromosome 18q loss is less frequently observed in breast cancer compared to other cancers and the apparent lack of intragenic mutations in the *Smad4* gene suggests that signaling mediated by *Smad4* in suppressing metastatic progression of breast cancer may arise primarily from alterations in other key mediators or effectors which participate in the execution of these signaling events. We propose to identify the mediator and effector genes, which regulate metastatic progression of breast cancer upon inactivation of the *Smad4* signaling pathway using appropriate tumor cell lines as well as experimentally developed derivative test and control cell lines as model systems.

2. Original tasks and the rationale for modifications in the experimental approach:

Although our original proposal was to primarily seek alternate target genes that are either localized to chromosome 18q or novel *Smad* genes involved in metastatic breast cancer, we reformulated our original goals to consider alternate modes of inactivation or loss of function of already known Smads as well as the regulatory and/or effector gene products of the central player, *Smad4*. The apparent lack of additional target genes localized to 18q21 prompted us to expand our focus to consider inactivation of overall *Smad4* signaling pathways due to the fact that disruption or unscheduled activation of critical genes mediating these events could also have

similar effect as direct targeting of the *Smad4* gene. The original tasks, modified expanded tasks and the justification for the slight shift in our original aims are as follows:

Task 1. Directed isolation of *SMAD* genes localized to 18q21 as potential target genes.

Expanded Task 1. Determination and identification of genetic and epigenetic alterations in all known and novel *Smads* as potential target genes.

Rationale: Although the TEGD (targeted expressed gene display) technique developed by us has not revealed any novel *Smads* to date, it has enabled the successful detection of previously unknown loss- or over-expression of the different *Smad* genes in breast tumors/ cell lines.

Task 2. Positional cloning of genes localized to chromosome 18q21.

Expanded Task 2. Identification of alternate target genes involving the *Smad4* signaling pathway.

Rationale: Despite the lack of inactivation of other known/ predicted genes localized to 18q21, the tissue specific inactivation of gene products that either regulate or mediate the effects of *Smad4*, the central player in the *Smad* signaling pathways, could lead to similar outcomes as inactivating the *Smad4* gene itself.

Task 3. Evaluation of candidate target genes. This task remains unmodified.

We have made substantial progress toward the identification of alternate targets for inactivation of the *Smad* signaling pathways in breast cancer. We have also laid the groundwork for the discovery of *Smad4* regulatory and responsive genes as alternate targets for inactivation/ activation in the mediation of metastatic breast cancer. Furthermore, we believe that these studies could shed light on the molecular basis of breast cancer metastasis.

3. Body: Procedures and progress report:

Evaluation of *Smad* alterations in breast cancer.

The analysis of genetic alterations in the various *Smad* genes in previous studies led us to conclude that mutations in *Smad2* and *Smad4*, localized to chromosome 18q, are rarely observed in breast cancer (1). Therefore, we decided to survey the differences in overall expression patterns of the various *Smads* in breast cancer using the TEGD technique. The TEGD analysis suggested to us that loss of expression of *Smad4* and *Smad8* and over-expression of the *Smad7* gene could be major targets for aberrant *Smad* signaling events in subsets of breast cancers (Figure 1). We decided to further validate these observations and determine the significance of this abnormality in breast cancer using semi quantitative RT-PCR to analyze the expression patterns using appropriate gene specific primer pairs (Figure 2). We plan to extend these studies to a larger set of breast cancer cell lines and tumors derived from various stages of cancer, and also probe for alternate modes of inactivation in addition to the analysis of gene expression. The initial step to determine gene inactivation will again be by semi quantitative RT-PCR (3; Figure 2). The presence or absence of expression, and the splice variants of the various *Smads* will be determined by this method. Subsequently, initial mutation searches will be conducted by IVSP analysis and by direct sequencing (3). We will also investigate whether the loss of expression of *Smads* is due to gene silencing by promoter DNA methylation.

Overall, the preliminary data so far suggested to us that over-expression of *Smad7* or loss of expression of the *Smad8* gene could be major targets for aberrant *Smad* signaling events in subsets of breast cancers (Figures 1 & 2). Based on these preliminary data, we plan to acquire commercially available antibodies or raise them to *Smad 7* and *Smad 8* and determine the feasibility of conducting

immunohistochemistry (IHC) on control and test cell lines to assess whether over- or under- expression respectively could eventually be applied to tumor samples (not within the scope of this proposal) for diagnostic and or prognostic evaluations.

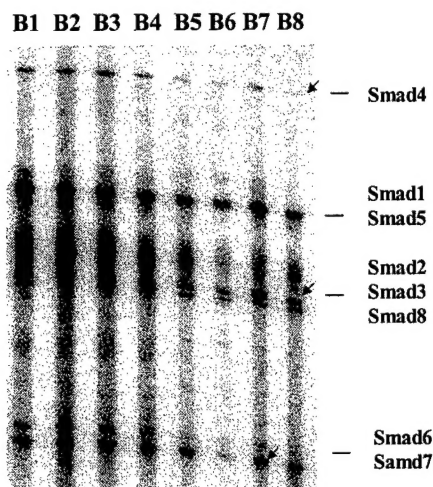


Figure 1. Targeted expressed gene display (TEGD) analysis of Smads in breast cancer.

PCR products for *SMADs* using degenerate primers were analyzed by TEGD. Lanes B1-8 correspond to PCR products generated using cDNA templates from the normal mammary tissue (B1) and tumor (B2-8) samples. The arrows point to distinct PCR products that were abnormal compared to the normal control. The positions of various *Smad* genes and their variants as identified from sequence analysis are indicated on the right panel.

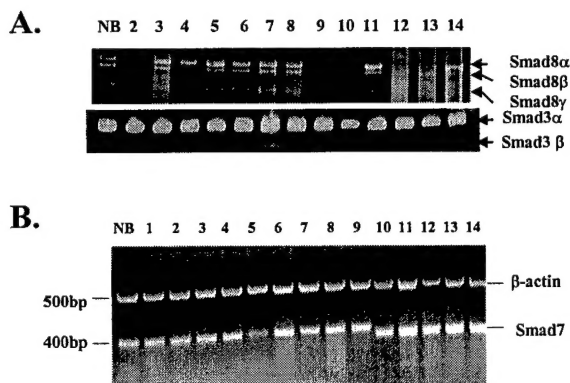


Figure 2. Semi-quantitative RT-PCR analyses of Smad expression in breast cancer.

Total RNA was prepared using the Trizol method from the indicated breast cancer samples and analyzed by RT-PCR (Lanes 1-14). NB refers to sample from normal breast tissue. *Smad3α* and *Smad3β* are two of the major differentially spliced forms of *Smad3*. *Smad8α*, *Smad8β* and *Smad8γ* are three of the major differentially spliced forms of *Smad8* which correspond to the full-length, deletion of exon 2, and deletions of exons 2&3, respectively. Analysis of the *β-Actin* gene is used for normalization and quantitation of the *Smad* genes.

Identification and evaluation of *Smad4* signaling mediator/ effector genes involved in metastatic breast cancer.

A recent study analyzing the pancreatic adenocarcinoma cell line Hs766T, which harbors a homozygous deletion of the *Smad4* gene, reported an increase in the expression of VEGF and a decrease in expression of TSP-1 (2). These observations suggested that disabling *Smad4* signaling events potentially plays a role in promoting the increased propensity for angiogenesis

and metastasis of cancer. We propose to test this phenomenon in breast cancer metastasis. We have chosen the Smad4 inactivated colon cancer cell line HCT116 (*Smad4*^{-/-}) that was experimentally generated to be null for Smad4 and a breast cancer cell line MDA-MB 231, which harbors a deletion of exon 5 in one allele and a point mutation (P303L) in the second allele, thus effectively inactivating the Smad4 gene. We have generated derivative stables, from these Smad4 null/inactive cell lines, that harbor wild-type Smad4 or their corresponding vector controls as the experimental model systems to determine the effect of Smad inactivation in metastatic breast cancer (Figure 3A). The ability to complement Smad4-mediated transactivation in these stable isogenic model cell lines has also been investigated using luciferase reporter assays to confirm the intactness of the Smad4 signaling pathway. These cell lines were transfected with pSBE4-Luc (Luciferase reporter with the Smad4 binding site) to determine whether the expression of Smad4 mediates reporter activity. Our preliminary experiments in which measurements made after 24 hours of culturing with and without TGF β suggest that Smad4 signaling could be reconstituted (Figure 3C).

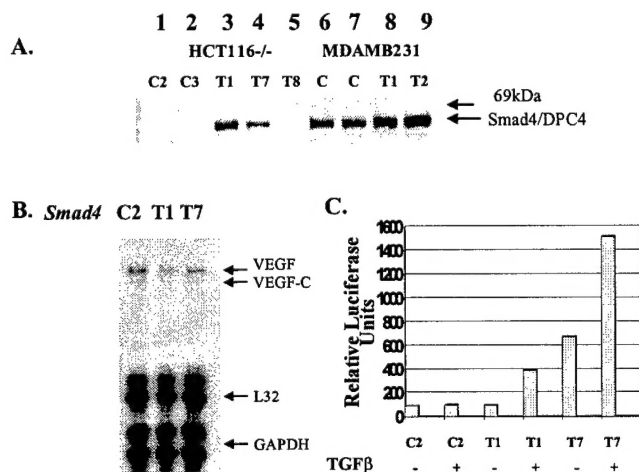


Figure 3. Relationship between *Smad4* status and the expression of VEGF.

A. Western blot analysis to screen for stable *Smad4* over-expression in test and control cell lines. Lanes 1&2 and 6&7 are stable transfectants of the pCMV vector while Lanes 3-5 and 8&9 are stable transfectants of the pCMV-*Smad4* of the indicated cell lines. **B.** Effect of over-expression of the *Smad4* gene on VEGF and VEGF-C. Total RNA was analyzed with the RiboQuant multi-probe template set (hAngio-1; BD-PharMingen, San Diego, CA) to detect the indicated mRNAs. L32 and GAPDH were included in each template set as internal controls. **C.** *Smad4* signaling as determined by *Smad4* responsive luciferase. pSBE4-Luc was transfected into the indicated stable cell lines and assessed for Luciferase activity after 24 hours of cell culture.

Furthermore, preliminary experiments are encouraging as the introduction of wild-type *Smad4* into the colon cancer cell line HCT116 with the deletion of *Smad4* exhibited a decrease in VEGF expression (Figure 3B). The lack of complete suppression of VEGF expression in these experiments could suggest that under the conditions of the experiment the competing pathways that promote VEGF expression could be still operative (e.g., HIF-1 α) in these cells despite the fact that *Smad4* could function as a major suppressor.

We are also in the process of carefully analyzing our breast cancer models and determining the identities of the various genes that may be differentially regulated and potentially participate in *Smad4* mediated processes in the metastatic progression of breast cancer. We plan to undertake the analysis of a wide spectrum of mediator/effector genes/ESTs for their up- or down-regulation using microarrays (Affymetrix).

Once legitimate metastasis mediator and effector gene(s) are identified, evaluation of the

status of the candidate gene(s) for inactivation/ activation in metastatic breast cancer will commence as described in the original proposal (3; Task 3).

4. Key research accomplishments:

Our unexpected preliminary finding that 30% of the breast cancers we sampled exhibited loss of Smad8 expression is very significant as it is equivalent to other established and highly valued markers such as *Her/neu*. The identification of target gene(s) that disable Smad signaling to promote breast cancer could potentially provide key arsenals to combat breast cancer.

We have identified/ generated appropriate tumor cell lines as well as experimentally developed derivative test and control cell lines as model systems to identify and isolate the metastatic breast cancer mediator and effector genes involving Smad4.

5. Conclusions:

- (1) Loss of expression of *Smad4* and *Smad8* and over-expression of the *Smad7* gene are potentially major mechanisms for inactivation of Smad signaling in breast cancer.
- (2) The loss of Smad8 expression in 30% of the breast cancers is very significant as it is equivalent to other established and highly valued tumor markers such as *Her/neu*.
- (3) The identification of target gene(s) that disable Smad signaling to promote breast cancer could potentially provide not only novel and valuable diagnostic and prognostic tumor markers but also key arsenals to combat breast cancer.

6. References:

1. Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGF β and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.
2. Schwarte-Waldhoff I, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, and Schmiegel W. 2000. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc. Natl. Acad. Sci.* 97: 9624-9629.
3. Thiagalingam, S. 2001. Molecular detection of Smad4/Smad2 alterations in colorectal tumors: Colorectal Cancer Methods and Protocols. In *Methods in Molecular Medicine*. S. M. Powell (Ed) Humana Press Inc., New Jersey, 50: 149-165.

7. Scientific presentations/ publications relevant to this grant:

Seminars by Dr. Sam Thiagalingam:

LOH: A predictor to map tumor suppressor genes involved in cancer and the molecular mechanisms of their occurrence. DDC Seminar, Medical College of Wisconsin, Milwaukee, October 15, 2001.

The Smad connection to cancer. Boston University School of Medicine Rheumatology Conference. September 4, 2002.

Publications:

Cheng, K-h., H. J. Lee, J. F. Ponte and S. Thiagalingam. 2002. Identification of alternate targets for the inactivation of Smad signaling in cancer using a novel method: Targeted Expressed Gene Display. *Manuscript in preparation*.

Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGF β and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.

Thiagalingam, S., R. L. Foy, K-h.Cheng, H. J. Lee, A. Thiagalingam, and J. F. Ponte. 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. *Current Opinion in Oncology* 14(1): 65-72.

Principal Investigator: Thiagalingam, Sam

APPENDIX I -Reprints

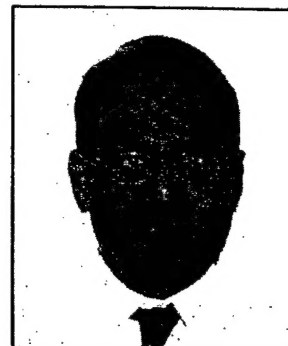
Relevant publications by the P. I.:

1. Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGF- β and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.
2. Thiagalingam, S., R. L. Foy, K-h.Cheng, H. J. Lee, A. Thiagalingam, and J. F. Ponte. 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. *Current Opinion in Oncology* 14(1): 65-72.

TGF β and its *Smad* Connection to Cancer

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Abstract: The resistance to growth inhibition commonly observed in a variety of TGF β disabled human cancers, the potential role of TGF β in the exacerbation of malignancy and the effects of TGF β in suppressing the immune system, all emphasize the importance of pathways mediated by this polypeptide to the neoplastic process. Early investigations to understand the molecular basis of cancer due to defects in TGF β signaling were concentrated on examining the abundance of biologically active TGF β and its binding to TGF β receptors. However, major breakthroughs in understanding the molecular basis of the TGF β mediated effects in cancer came from genetic evidence for inactivation of the various players in its signaling cascade. The vast majority of current evidence is derived from the identification of mutations causing structural defects in TGF β receptors and *Smad* genes, the downstream effectors of the TGF β signaling pathway that have emerged from the analysis of human cancers. The involvement of Smads at the receptor level upon activation by a TGF β bound receptor, their participation in signal transmission to the nucleus and their direct roles in the regulation of target genes have made the various *Smad* genes critical targets for inactivation of TGF β signaling in cancer. To date, eight human homologues of the *Smad* genes have been identified and are classified into three distinct classes based on their structure and function. In this review, we discuss TGF β signaling *via* the Smads and the known and predicted points at which TGF β signaling could become altered in human cancer.

INTRODUCTION

The signaling pathways mediated by the members of the transforming growth factor-beta (TGF β) family are implicated in a number of biological processes including cell differentiation and proliferation, determination of cell fate during embryogenesis, cell adhesion, cell death, angiogenesis, metastasis and immunosuppression [1-4]. Due to the wide array of functional consequences mediated by its signaling events, TGF β could impact tumorigenesis by affecting any one or a combination of the following processes: (1) altering the delicate balance between cellular proliferation and apoptosis; (2) affecting induction of extracellular matrix proteins such as proteoglycans, collagens, fibronectin, laminin, tenascin and vitronectin and regulating their breakdown by extracellular proteases and metalloproteinases by controlling the induction of their inhibitors such as plasminogen activator inhibitor (PAI-1) and tissue inhibitor of metalloproteinase (TIMP); and (3) disabling the tumoricidal activity or cytokine production of the immune system [5-11]. The major mechanisms that disrupt the carefully regulated balance of these events may consist of changes in the induction of gene expression patterns and in the functionality of proteins, which are affected by internal or external cues or familial and/or sporadic genetic changes. Although there has been significant progress in unraveling some of the genetic and epigenetic

alterations that underlie these overall abnormalities in signaling by TGF β , the nature and cues prompting changes that occur at the level of regulation of gene expression, protein synthesis and/or post-translational modifications will most likely be elucidated only by the efficient application of high throughput methodologies such as gene expression microarrays and proteomics.

TGF- β SIGNALING

Although TGF β was originally discovered for its positive role in transformation and tumor progression, most of the recent efforts have focused on the understanding of the mechanism of epithelial cell growth inhibition [12-14]. The TGF β family of factors is comprised of nine subfamilies/members with homology at the amino acid level ranging from 23 to 92%. The BMP (Bone Morphogenetic Protein), activin and TGF β subfamilies have been the most widely studied. The TGF β family members bind to specific receptors, which consist of two major subfamilies, type I (RI) and type II (RII) receptors. These receptors are structurally similar and have cysteine-rich extracellular regions and an intracellular kinase domain with serine/threonine kinase activity [15,16]. The RI receptor has a conserved 30 amino acid segment adjacent to the kinase domain rich in glycine and serines known as the GS region which forms a wedge against the catalytic center [17]. TGF β factors, which are dimers, bring together the RII and RI receptors into a heterotetrameric complex. The RII receptor's kinase domain becomes constitutively active and phosphorylates the GS domain of the RI receptor. Activated RI receptors can then mediate their activities through the

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Smad proteins. The role of vertebrate Smad (Sma and Mad) proteins in TGF β signaling was predicted from their high level of homology to the Mad (Mothers against dpp) from *Drosophila* and the Sma-2, Sma-3 and Sma-4 proteins from *Caenorhabditis elegans* in analogous signaling pathways [18-22].

To date at least eight homologues of the *Smad* genes have been identified and shown to be downstream of the serine/threonine kinase receptors (Table 1, Fig. 1). Smads are molecules with a relative mass of 42Kd-60Kd composed of two regions of homology (Mad Homology (MH) domains) at the amino and carboxy terminals of the protein termed the MH1 and MH2 domains, respectively, which are separated by a proline rich acidic linker region of variable length and sequence [34-36]. The amino terminal MH1 domains share 40-94% homology among Smads while the carboxy terminal MH2 domains are 38-98% homologous in their amino acid sequence (Fig. 1), [35]. The MH2 domain is involved in homo and heteromeric complex formation, as well as in transcriptional activation and repression, whereas the MH1 domain has DNA binding activity [36-38]. The MH2 domain of Smad2 and Smad3 interacts with the RI receptor [39]. Prior to activation of the receptor-regulated Smads by receptors, MH1 and MH2 domains interact with each other resulting in auto-inhibition [40,41].

The Smad family of proteins is divided into three distinct classes based on their structure and function [12]. The first category consists of pathway-restricted or receptor regulated Smads (R-Smads): Smad1, Smad5 and Smad8 (also known as MADH6/Smad9 in humans), which are specifically involved in BMP signaling whereas Smad2 and Smad3 are TGF β /activin pathway restricted. These Smads are directly phosphorylated by RI receptors after phosphorylation of the RI receptor by the RII receptor. The pathway restricted Smads have a characteristic Ser-Ser-X-Ser (SSXS) motif in their C-terminal region. The two-most C-terminal serine residues of these Smads are phosphorylated by RI receptors [42-44]. Phosphorylation at these sites has been shown to be necessary for Smad2 to interact with the second class of Smads known as the common mediator Smad (Co-Smad) [45]. Smad4 is the only member of this class of Smads

known in mammals. However, the recent identification of two Smad4 proteins (Xsmad4 α and Xsmad4 β) in *Xenopus* opens up the possibility that homologues of Smad4 may exist in mammals [46]. Smad4 lacks the C-terminal SSXS motif and is not phosphorylated by RI receptors [41]. Smad4 is involved in all distinct pathways and plays a central role by forming heteromeric complexes with the R-Smads.

SARA (Smad anchor for receptor activation) is a FYVE domain protein that only interacts with the MH2 domains of Smad2 and Smad3 [47]. SARA exhibits preferential binding to unphosphorylated Smad2 and becomes released when Smad2 is phosphorylated by the TGF β RI receptor. The Smad2-SARA and Smad2-Smad4 complexes apparently exist in a mutually exclusive manner. The double zinc finger FYVE domain of SARA is believed to be involved in directing the localization of R-Smads to the membrane where the TGF β RI receptors are located and thus increasing the effective concentration of Smad signaling molecules in their vicinity. Once the R-Smad molecules become phosphorylated and released from the SARA adaptor protein, phosphorylated R-Smads form R-Smad-Co-Smad complexes that translocate from the cytoplasm to the nucleus.

The crystal structure of the Smad4 MH2 domain has been resolved [35]. The monomer contains a β -sheet sandwich, capped at one end with a group of three large loops and an α -helix (loop-helix domain) and at the other end by a triple α -helix structure. These monomers assemble to form a trimeric structure in the crystal with the loop helix domain of one monomer interacting with the triple helix of the next; the resulting structure resembles a disk. On the face of the disk opposite the amino terminal side, the third loop (L3) from the loop-helix is exposed on the surface. It has been suggested that this loop is critical in mediating the formation of a hexameric complex between Smad4 trimers and trimers of phosphorylated R-Smads [48,49]. In addition to these predictions based on crystal structures, other models for the nature of Co-Smad and R-Smad hetero-oligomeric complexes have been proposed from biochemical and structural studies, which include heterohexamer, heterotrimer and heterodimer formations. [48-53]. At the time of this writing, the most recent biochemical and functional data

Table 1. Human SMAD Genes and Cancers

Gene	Map position	Alterations in cancers	Reference(s)
<i>SMAD1</i>	4q28-31	Not detected	[23]
<i>SMAD2</i>	18q21	Colon, liver and lung	[23,24,25]
<i>SMAD3</i>	15q21-22	Not detected	[23,26]
<i>SMAD4</i>	18q21	Colon, head & neck, leukemias, liver, lung, ovary and pancreas	[21,23,26-31,151]
<i>SMAD5</i>	5q31	Not detected	[23,26]
<i>SMAD6</i>	15q21-22	Not detected	[23,26]
<i>SMAD7</i>	18q21	Not detected	[23,26,32]
<i>SMAD8</i>	13q12-14	Not detected	[33]

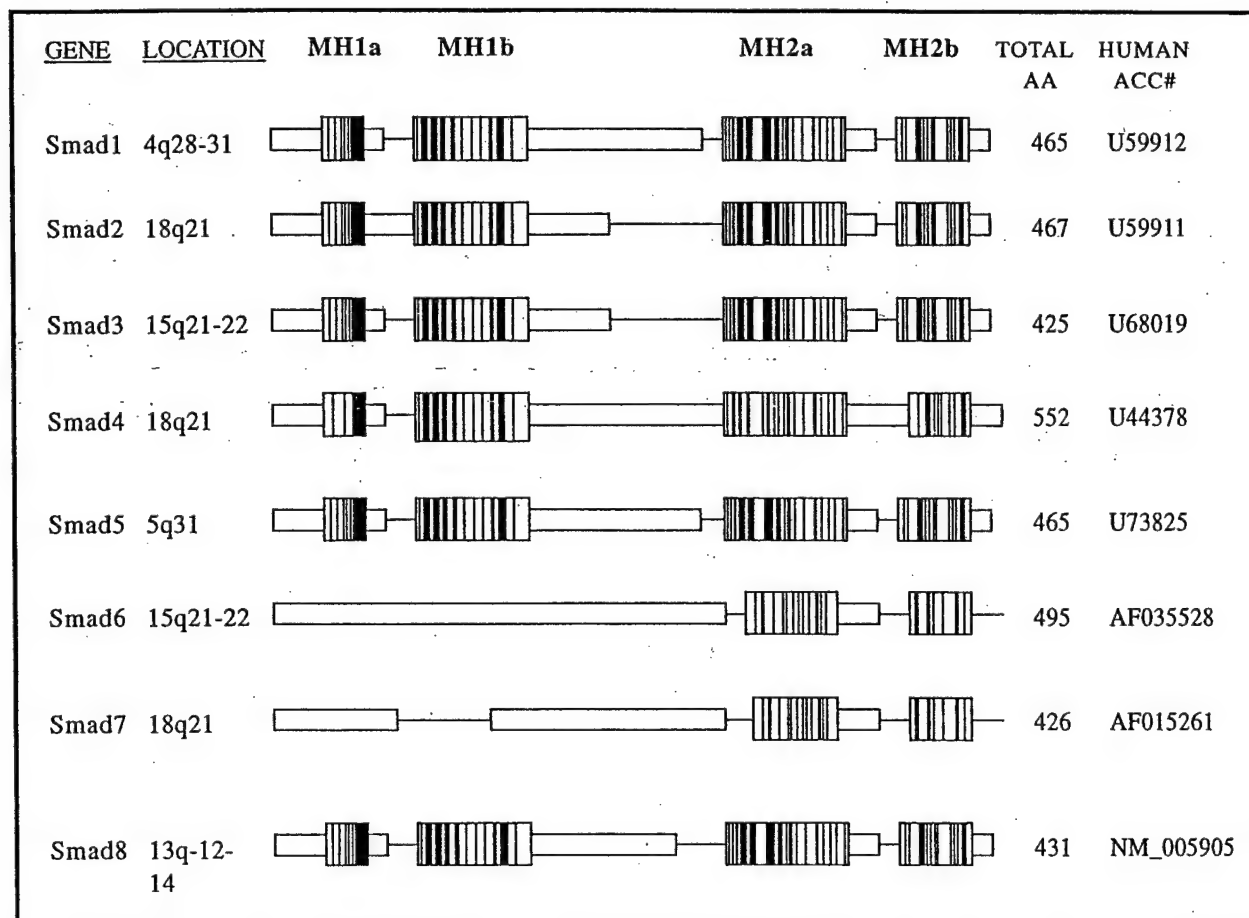


Fig. (1). Amino acid alignment of the known human Smad homologues.

Mad homology (MH) domains, labeled MH1a, MH1b, MH2a and MH2b, represent stretches of sequence that are highly related in Smad proteins. The thin vertical lines denote identical residues in at least five genes. The horizontal lines signify gaps introduced in order to optimize the alignment. Using Smad1 as a reference, MH1a, MH1b, MH2a and MH2b extend from codons 20 to 45, 68 to 145, 265 to 367, and 402 to 454, respectively. The inhibitory Smads, Smad6 and Smad7, do not possess MH1 domains, but the two of them do share regions of homology in their N-termini. The chromosomal position is shown in the second column, and accession numbers for the human Smad genes are shown in the last column.

strongly support the existence of a heterodimer between a Co-Smad and a R-Smad as the predominant basic functional unit [52,53].

The structural analysis of the Smad3 MH1 domain showed that the domain adopts a compact globular fold, with four α -helices, six short β strands, and five loops. A β -hairpin loop mediates the direct contact with DNA in the major groove. The β -hairpin loop is comprised of residues Leu-75, Gln-76, and Val-77 of strand B2, residues Arg-80, Lys-81, and Gly-82 of B3, and two connecting residues, Ser-78 and His-79. The β -hairpin is among the most highly conserved regions in Smad proteins and all the residues except the two at the turn of the β -hairpin are invariant among mammalian Smads [35]. Smad3 and Smad4 bind to DNA via the MH1 domain at sequences that contain AGAC, called Smad Binding Elements (SBEs) [55-57]. It has also been shown that an extra stretch of amino acids N-terminal to the β -hairpin loop present in Smad2 (derived from exon 3)

affords steric hindrance which inhibits DNA binding of the homo-oligomer of Smad2 [35,55,58].

The regulation of Smad signaling could occur at several levels including direct effects on Smad molecules affecting their functional status or by determining their stability or half-life. Upon translocation into the nucleus, each of the R-Smad-Co-Smad complexes could activate a specific set of genes through cooperative interactions with DNA and other Smad interacting DNA-binding proteins (SIDBP) and/or cofactors. R-Smad translocation to the nucleus is not dependent upon Smad4, but Smad4 translocation into the nucleus requires an activated R-Smad [59,60]. It has been shown that the MH1 and MH2 domains of Smads interact with a number of factors in the nucleus to determine the specificity of gene activation or repression (Table 2) [61,62]. This specificity for DNA binding of Smad transcription factor complexes to regulatory regions of genes would theoretically lead to transcriptional activation or repression

Table 2. Cofactors of Smad Transcriptional Regulation

Co-Factor	Smad/ Domain Contacted	Molecular Basis of Activity	Reference(s)
Activators			
AML/PEBP2/CBFA	Smad2, 3 MH1, MH2	Cooperate with Smads to induce gene expression	[82-84]
ATF-2	Smad3 MH1	"	[85]
Mixer/milk	Smad2 MH2	"	[86]
Fast1/Fast2	Smad2, 3 MH2	"	[63, 64, 86]
c-Fos	Smad3 MH2	"	[87]
c-Jun, JunB, JunD	Smad3 MH1, Linker	"	[87-89]
SP1	Smad2, 3, 4 MH1 and/or MH2	"	[90-94]
CBP and p300	Smad2, 3 MH2; Smad4 activation domain	Opens chromatin structure by histone acetylation	[55, 57, 88, 95-98]
MSG1	Smad4 MH2	Interacts with CBP and p300	[99, 100]
Repressors			
AML/ETO	Smad2, 3 MH2	Unknown	[101]
SIP1	Smad2, 3 MH2	Unknown	[74, 81]
TGIF	Smad2, MH2	Recruits histone deacetylases	[38, 75]
E1A	Smad3 MH2	Prevents interaction with CBP and p300	[102]
Evi-1	Smad3 MH2	Prevents Smad3 binding to DNA	[103, 104]
Ski, SnoN	Smad2, 3, 4 MH2	Recruit repressors, N-CoR, mSin3 and histone deacetylases. Bind DNA	[37, 77-80]

of a unique set of gene(s) with a high level of specificity for the tissue and/or the overall physiological signal being transduced. The binding of an activated Smad transcription complex to specific regulatory regions and transactivation may be defined in part by subsequent SIDBPs such as FAST1, FAST2 and Jun/Fos [59, 63-67]. On the other hand, transcriptional specificity could also occur by recruitment of factors interacting with Smads by protein-protein interactions such as in the cases of p300 and CBP (CREB-binding protein) [68-71]. p300 and CBP are histone acetyl transferases (HAT) that increase transcriptional activation due to their ability to alter the nucleosome structure *via* chromatin remodeling by hyperacetylation of neighboring chromatin and by recruitment of the RNA polymerase holoenzyme to the promoter [72,73]. Similarly, DNA binding factors such as SIP1 and TGIF or non-DNA binding factors such as Evi1, Ski and SnoN could interact with the Smad proteins in the transcription complex leading to the repression of transcription [74-81]. The homeodomain protein TGIF (TG interacting factor) is believed to mediate Smad-dependent repression of transcription either by recruiting histone deacetylases (HDACs) or by competing with p300 or CBP for association with Smads [1,75]. Alterations in the relative levels of the coactivators or corepressors in the cell depending on the tissue type and/or physiological state would ultimately determine whether TGF β signaling would result in activation or repression of specific genes.

The third class of Smads includes Smad6 and Smad7 which were identified as anti-Smads or inhibitory Smads (I-Smad) due to their ability to act as inhibitors of TGF β signaling. Smad6 and Smad7 are rapidly induced by TGF β -like molecules, IFN γ , and a number of other growth factors and cytokines and they elicit negative feedback regulation of Smad signaling pathways. Smad6 and Smad7 interact with activated RI receptors with high affinity to prevent access to RI receptors by R-Smads and thus inhibit phosphorylation of R-Smads [32,105,106]. STRAP (serine-threonine kinase receptor associated protein) contains a WD domain and associates with Smad7 as well as with the RI receptors augmenting the inhibitory effects of Smad7 [90,107]. Furthermore, it has been postulated that Smad6 may also compete with Smad4 for association with activated Smad1 in BMP signaling, and may possibly function as a co-repressor for transcription *via* its interaction with Hoxc-8, a homeodomain protein factor [108].

The ubiquitin proteasome pathway is implicated in the turnover of Smads as well as TGF β receptors in the regulation of the TGF β signaling pathway. Smurf1 and Smurf2, E3-ubiquitin protein ligases, target R-Smads of the BMP signaling pathway (Smad1 and 5), Smad7 and TGF β receptors respectively [109,110]. Smad7 expression is stimulated by IFN- γ and promotes Smurf2-Smad7 complex formation in the nucleus; this complex is translocated to the cytoplasm and eventually binds to the TGF β receptors at the

plasma membrane leading to their degradation [110]. Poly-ubiquitination of Smad2 and subsequent degradation by the proteasome has also been reported suggesting that regulation of the TGF β signaling components occur through the ubiquitin proteasome pathway [111].

Additional detailed accounts of Smad signaling can also be found in some of these recent reviews [2,3,112,113]. A heuristic model illustrating the different roles of the Smads in TGF β signaling is outlined in Fig. (2).

ABUNDANCE OF BIOLOGICALLY ACTIVE TGF β IN CANCER

TGF β ligands secreted in their latent forms are approximately 390 to 414 amino acids long and consists of a latent associated peptide (LAP) region at the amino-terminal and the biologically active mature form of TGF β at the carboxy-terminal ends [114]. Proteolytic processing in the Golgi apparatus by the convertase family of endoproteases and conformational changes assisted by LAP are believed to be necessary to form the biologically active form of TGF β .

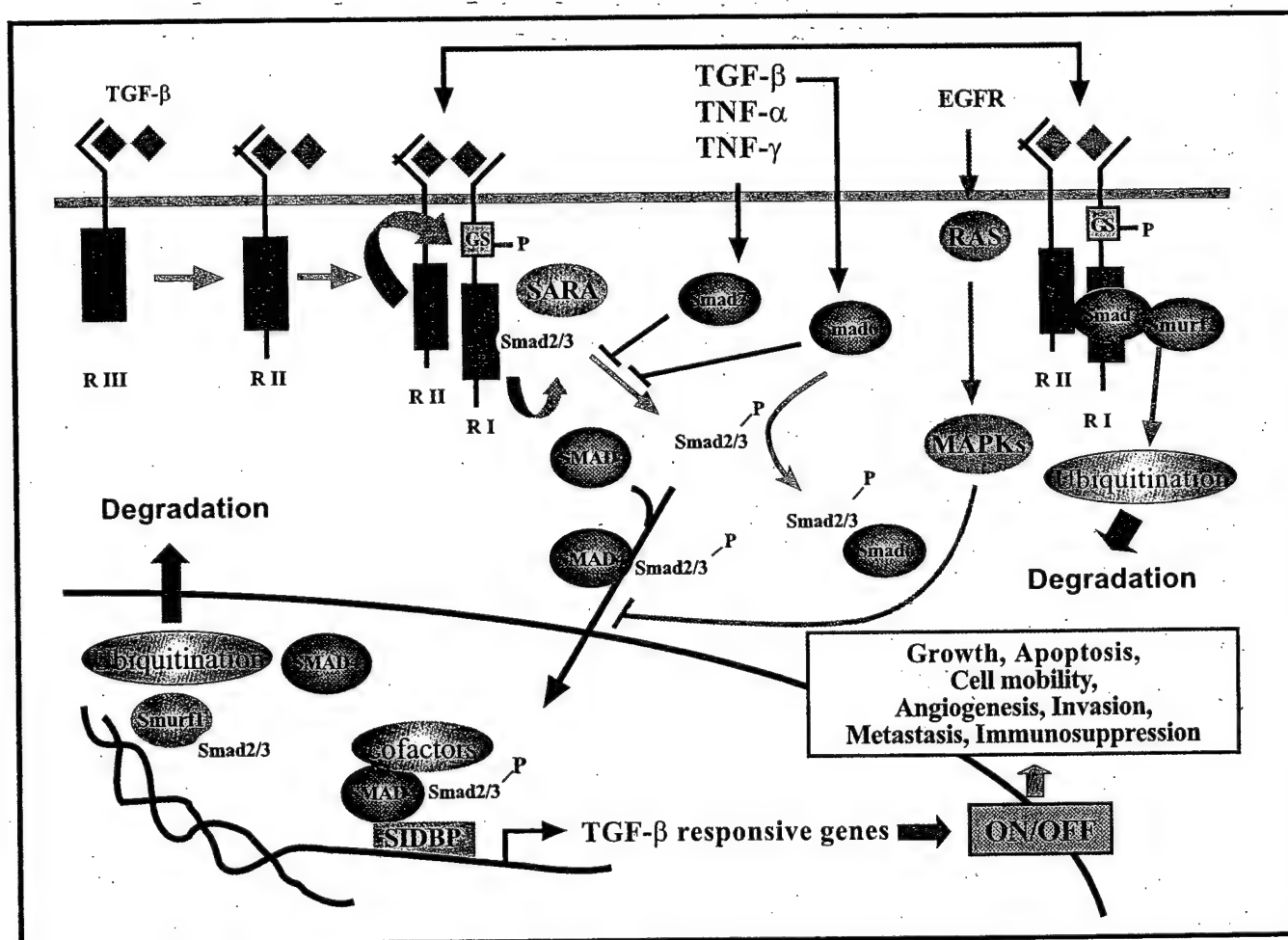


Fig. (2). A model for the Smad connection to the TGF β signaling pathway.

TGF β binds to a type-III receptor, which helps to increase the localized effective concentration of the ligand in the proximity of the type-II receptor (RII). The TGF β ligand binds to the RII which phosphorylates a type I receptor (RI) that in turn initiates signaling via the Smad proteins. The activated RI recognizes receptor regulated Smads (R-Smad), such as Smad2 or Smad3 and phosphorylates them at specific carboxy-terminal serine residues. These phosphorylated R-Smads form a heteromeric complex with the common-mediator Smad (Co-Smad), Smad 4, and the complex is translocated into the nucleus. In the nucleus, the R-Smad/ Co-Smad hetero-oligomer, either by itself or by associating with heterologous Smad interacting DNA binding proteins (SIDBPs), such as FAST-1, and/or other cofactors, mediate specific transcriptional activation or repression responses. The inhibitory Smads (I-Smads), such as Smad6 and Smad7, are able to compete with R-Smads by stably binding the RI or by preventing association of R-Smads with Co-Smads, effectively blocking downstream signaling events. There are numerous other signaling pathways such as Ras-MEK that could modulate the end effects by establishing cross talk among the different pathways. The ubiquitin-proteasome pathway continuously recycles the various players in the TGF β signaling pathway, thus contributing to the inducibility of the system. Please refer to the text for more details.

LAP remains noncovalently linked to the bioactive TGF β and the TGF β -LAP complex is apparently bound by latent TGF β binding proteins (LTBP) to stabilize the protein, ensure correct folding of TGF β and to enhance secretion of TGF β to the extracellular matrix of target cells for storage or bioactivity. It has been reported that LTBP was absent in malignant but not in benign prostatic tissue suggesting that tumorigenicity promoted by TGF β could be regulated at the ligand level [115]. The other molecules, which could play critical roles in determining the levels of free and mature forms of TGF- β available to initiate signaling, are thrombospondin-1 (TSP-1), endoglin (CD105) and betaglycan (TGF β receptor type III (TGF β R III)). TSP-1 is suggested to play an important role in the activation of TGF β by inducing conformational changes in LAP, which prevents reassociation between LAP and mature TGF β [116,117]. Endoglin exhibits strong homology to betaglycan in the transmembrane and intracellular domains and binds to TGF β . It is reported to be overexpressed in breast and lung cancers with increased angiogenesis and decreased TGF β responses [118-121]. On the other hand, overexpression of the TGF β RIII is associated with enhanced TGF β responsiveness due to its ability to act as a reservoir for active TGF β ligands. TGF β RIII has high affinity binding sites for TGF β ligands and facilitates their interaction with TGF β RII [122-124]. Under some conditions increased expression of TGF β RIII has been associated with reduced tumorigenicity, however, it is believed to enhance tumorigenicity under other conditions [121]. The increase in tumorigenicity observed under these latter conditions may be associated with metastatic conversion of tumor cells in advanced cancer.

TGF β RECEPTOR DEFECTS IN CANCER

Resistance to TGF β mediated growth inhibition is also found to be common in a variety of human cancers, which emphasizes the importance of pathways mediated by this polypeptide to the neoplastic process [125-127]. The early investigations to understand the molecular basis of this resistance were concentrated at the level of TGF β RI and RII, the serine/threonine kinase receptors. A correlation between resistance to TGF β growth inhibition due to lack of TGF β receptor expression was established and reported in a variety of human cancer cell lines [128-131]. The first genetic evidence for the inactivation of the TGF β signaling pathway due to mutations causing structural defects in the TGF β RII revolutionized the understanding of the molecular basis of this defect [129,131,132]. RII mutations were initially reported in colon cancer with microsatellite instability (MSI) resulting from frame shifts clustered in a naturally occurring 10 bp microsatellite-like polyadenine tract in the 5' coding half of the gene. Subsequent studies demonstrated that additional sites such as residues in the kinase domain could also be inactivated in both MSI and non-MSI colon tumors [131-133]. Microsatellite instability in this polyadenine repeat, referred to as the BAT-RII region, leads to frameshift mutations resulting in truncated receptors that lack kinase domains and are thus functionally inactive [131]. In addition to colorectal cancers, mutations in the BAT-RII region are also found in gastric cancers and in gliomas, but rarely in microsatellite unstable tumors of the

pancreas, liver, endometrium and breast [131,132,134]. Furthermore, most of the non-BAT-RII mutations are centered in the kinase domain and affect the ability of the RII receptor to phosphorylate the RI receptor [133]. The analysis of a mutation, Thr315Met, in the kinase domain of the RII receptor exhibited the separability of end effects mediated by TGF β signaling, which may be important in cancer. This mutation in HNPCC kindred retained the ability to induce extracellular matrix proteins and PAI-1 characteristic of the metastatic potential while it lost its ability to induce p15 leading to the lack of TGF β mediated growth inhibition [135].

There is also evidence that TGF β RI could become inactivated in a subset of other cancers [127]. It has been reported that 33% of ovarian cancers have an inactivating mutation in the RI receptor while no mutations were observed in the type II receptor in the same tumor cohort [136]. Additionally, deletions of the RI receptor have been reported to occur at a low frequency in pancreatic and biliary carcinomas as well as in T cell lymphomas while mutations of specific amino acid residues were found in breast cancer [137-139]. A recent analysis of gastric cancer cell lines suggests that silencing of TGF β RI expression could also occur due to hypermethylation of a CpG island in the promoter region adding another mechanism of regulation that determines the abundance of TGF β receptors [140]. However these alterations alone do not explain the mechanism of inactivation of the TGF- β signaling pathway in an overwhelming number of tumors that are resistant to TGF β signaling effects.

INACTIVATION OF SMADS IN CANCER

The recent discovery of the *Smad* genes as downstream effectors of the TGF β signaling pathway and the frequent occurrence of mutations in these genes could be regarded as a major breakthrough in the understanding of the molecular basis of insensitivity and/or deregulation of TGF β mediated effects [21,23,27,28,141,142]. The isolation of the *Smad4* gene itself was based on the identification of target tumor suppressor genes localized to frequent homozygous deletions affecting 18q21.1 in pancreatic carcinomas [21]. Allelic loss of 18q21.1 has been associated with cancers of higher mortality and an increased risk of metastatic spread [27,143,144]. In addition to pancreatic cancer, *Smad4* mutations were also found in a subset of colon and lung cancers but rarely in others (Table 3) [23,27,28,141,142]. Two novel *Smad* genes, in addition to *Smad4*, have been isolated and localized to chromosome 18q21 (Table 1) [24,25,32]. Soon after the discovery of *Smad4*, *Smad2* was also localized to 18q21 and considered as a legitimate alternate candidate tumor suppressor gene localized to this region. Mutational analysis of *Smad2* by others and by us revealed that it is also inactivated in additional colorectal tumors [24,25]. In our study, we extended the analysis of *Smad4* inactivation by using the same set of tumors with LOH at 18q21 and found that some of the tumors, which lacked *Smad4* mutations harbored *Smad2* mutations (Table 4) [21,24,142]. These observations further strengthened our hypothesis that these two genes and other genes involved in TGF- β signaling may be alternatively targeted for

Table 3. Mutations in *Smad4*

Codon	Mutation	Predicted Change	Cancer	Reference(s)
43	TTG to TCG	Leu to Ser	Pancreas	[26]
50-51	A insertion	Frameshift/Stop	Colon	[150]
51	AAA to AAG	Lys to Lys (Silent)	Ovary	[151]
65	GGG to GTG	Gly to Val	Colon	[152]
95	TAT to AAT	Tyr to Asn	Colon	[150]
100	AGG to ACG	Arg to Thr	Pancreas	[28]
102	CCT to CTT	Pro to Leu	AML	[153]
115	TGT to CGT	Cys to Arg	Colon	[154]
129	AAT to AAG	Asn to Lys	Colon	[150]
130	GTC to GAC	Pro to Ser	Colon	[142]
162	2bp deletion	Frameshift/ Stop	Pancreas	[26]
168	GGA to TGA	Gly to Stop	Colon	[154]
195	TAC to TAA	Tyr to Stop	Colon, Pancreas	[26,154]
202-203	4bp deletion	Frameshift/ Stop	Lung	[141]
269-270	ACT to ACTT	Frameshift/ Stop	Colon	[154]
317	CAT to CGT	His to Arg	Ovary	[151]
326	1bp deletion	Frameshift/Stop	Colon	[150]
332	GAT to GGT	Asp to Gly	Liver (HCC)	[29]
336-338	2bp deletion	Frameshift/ Stop	Colon (HNPCC)	[154]
339-343	15bp deletion	Frameshift	Colon	[154]
343	TCA to TGA	Ser to Stop	Pancreas	[28]
343	2bp deletion	Frameshift/ Stop	Pancreas	[28]
351	GAT to CAT	Asp to His	Pancreas, Ovary	[28,151]
355	GAC to GAA	Asp to Glu	Colon	[150]
358	GGA to TGA	Gly to Stop	Colon, Pancreas	[21,142]
361	CGC to TGC	Arg to Cys	Colon	[142]
361	CGC to CAC	Arg to His	Colon	[154]
363	TGT to AGT	Cys to Ser	Colon	[154]
369	AAT to GAT	Asn to Asp	Pancreas	[155]
370	GTC to GAC	Val to Asp	Colon	[142]
379	GCA to ACA	His to Arg	Ovary	[151]
386	GGT to TGT	Gly to Cys	Ovary	[151]
401	TGC to CGC	Cys to Arg	Liver (HCC)	[29]
406	GCG to ACG	Ala to Thr	Pancreas	[26]
412	TAC to TAG	Tyr to Stop	Pancreas	[21]
415-416	4bp deletion	Frameshift/Stop	Colon	[154]
420	CGT to CAT	Arg to His	Lung	[141]

(Table 3) contd....

Codon	Mutation	Predicted Change	Cancer	Reference(s)
441	CGT to CCT	Arg to Pro	Lung	[141]
442	CAG to TAG	Gln to Stop	Colon	[154]
445	CGA to TGA	Arg to Stop	Colon	[150,154]
447-455	25bp deletion	Frameshift/Stop	Colon	[154]
450-459	28bp deletion	Frameshift/Stop	Colon	[154]
457	GCA to TCA	Ala to Ser	Pancreas	[155]
483	AGT to AAT	Aberrant splicing	Pancreas	[21]
483	4bp insertion	Frameshift/Stop	AML	[153]
492	1bp insertion	Frameshift/Stop	Seminoma germ cell	[30]
493	GAT to CAT	Asp to His	Pancreas	[21]
497	CGC to CAC	Arg to His	Colon	[154]
504	AGT to AGA	Ser to Arg	Ovary	[151]
507	AAA to CAA	Lys to Gln	Colon	[154]
515	AGA to GGA	Arg to Gly	Colon	[154]
515	AGA to TGA	Arg to Stop	Pancreas	[21,155]
516	CAG to TAG	Gln to Stop	Colitis	[156]
516-518	8bp deletion	Frameshift/ Stop	Pancreas	[21]
525	ATT to GTT	Ile to Val	Head and Neck	[157]
526	GAA to TAA	Glu to Stop	Head and Neck	[157]
528/529	4bp deletion	Frameshift/ Stop	Pancreas	[155]
538	GAA to TAA	Glu to stop	Colon	[150]
540-542	7bp deletion	Frameshift/ Stop	Colon	[154]

Table 4. Mutations in *Smad2*

Codon	Mutation	Predicted change	Cancer	Reference(s)
133	CGC to TGC	Arg to Cys	Colon	[25]
345-358	42bp in deletion	In frame deletion	Colon	[24]
346	TTT to GTT	Phe to Val	Colon	[154]
407	CAG to CGG	Gln to Arg	Liver (HCC)	[29]
431-454	9bp in deletion	In frame deletion	Lung	[27]
441	CAT to CGT	His to Arg	Colon	[158]
440	CTT to CGT	Leu to Arg	Colon	[25]
445	CCT to CAT	Pro to His	Colon	[25]
450	GAC to GAG	Asp to Glu	Colon	[25]
450	GAC to CAC	Asp to His	Lung	[27]

inactivation in cancer. Although homozygous deletion of 18q21.1 is also a major mechanism of *Smad2* and/or *Smad4* inactivation, specific mutations of these genes results in defects in their functionality. Many of these mutations are found in the MH2 domain and can dysregulate Smad signaling by: (1) preventing phosphorylation, (2) weakening interactions during homo and hetero-oligomerization, and (3) altering interaction with other factors affecting transcription of Smad regulated genes [11, 49, 145, 146].

The mutations that map to the interface regions between the Smad4 monomers destabilize the trimeric complex. Mutations in the third loop on the face of the disk disrupt the formation of heteromeric complexes whereas mutations in the hydrophobic core destabilize the structure of the protein [48]. In a study assessing the role of the Smad2 P445H mutation, it was shown that upon over-expression of Smad2 P445H, the mutant protein could become phosphorylated by the RI receptor and associate with Smad3 or Smad4, but was unable to disassociate from the receptor. Moreover, when the mutant protein was phosphorylated upon ligand binding, Smad2P445H bound stably with wild type Smad2 and blocked the nuclear accumulation of Smad2 and subsequent Smad2 dependent transcription [147]. It was also shown that a missense mutation in a conserved amino acid in the MH1 domain of both Smad2 and Smad4 resulted in an enhanced autoinhibitory interaction between the MH1 and MH2 domains, which decreased protein stability [34]. Mutations in the MH1 domain of Smad4 were found to eliminate its ability to bind DNA even though they still retained the ability to form complexes with Smad3. Additionally, these mutant proteins exhibit decreased protein stability and an impaired ability to translocate to the nucleus [148]. Finally, a novel mechanism of inhibition of TGF β signaling has been observed in mutations of a conserved residue of the MH1 domain of Smad2 and Smad4 which targets the mutant protein for rapid degradation via the ubiquitin-proteasome pathway [149].

The third *Smad* family member localized to 18q21, *Smad7*, is an important regulator of Smad signaling pathways primarily by antagonizing TGF β signaling via inhibition of R-Smads. Increased expression of Smad7 was shown in some pancreatic cancers, but there has been no evidence presented thus far for the existence of activating mutations or amplifications of the *Smad7* gene [26,159]. The clustering of the *Smad2*, *Smad4* and *Smad7* genes in a critical region of chromosome 18q21 which is deleted at high frequency in advanced tumors raises an intriguing possibility that there may be additional *Smad* genes localized to this region in addition to potentially unrelated tumor suppressor genes [11,24,142]. Similarly, *Smad3* and *Smad6* are located in the same general region on chromosome 15q21-22, which is frequently lost in a subset of breast, colorectal, lung and pancreatic tumors [160-162]. However, neither inactivating mutations, homozygous deletions, nor amplifications of *Smad3* or *Smad6* have been reported to date in human cancers [26,136,154,163-166].

Interestingly, the association of chromosome 18q loss [deletion of *Smad* gene(s)] with an advanced stage of human cancer and the observation of an increased malignant conversion frequency and decreased carcinoma latency in

mice with disabled TGF β signaling due to over-expression of the dominant negative TGF β RII, illustrates that the inactivation of TGF β signaling is a critical late event in the multi-step cancer progression [142,167-171]. Consistent with these studies are the recent findings that TGF β signaling may have a role in metastasis as discussed in a subsequent section of this review.

GROWTH INHIBITION AND APOPTOSIS

TGF β can inhibit cellular proliferation in a number of cell types. This inhibition occurs at the G₁ stage of the cell cycle and is accomplished through either one of the two following mechanisms: induction of cdk inhibitors such as p15, p21 and p27 or downregulation of Myc. p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d} are all members of the INK4 family of cyclin dependent kinase inhibitors that bind cdk4 and cdk6, to suppress their catalytic activity by preventing association with cyclin D [172]. TGF β signaling mediated p15 induction and activation was originally shown in keratinocytes and was later implicated in stabilizing p15, enhancing the formation of p15-cdk4 complexes and inhibiting cyclinD-cdk4 complexes in mammary epithelial cells [173,174]. It has been recently reported that Smad2, Smad3, Smad4 and Sp1 are directly involved in p15 induction confirming previous reports that had implicated Sp1 and Smad3 in p15 induction [91,175,176]. Early in G₁, cyclinD and cdk4/6 come together to form complexes that sense mitogens. p15 binds to cdk4/6 and inactivates its catalytic activity, which prevents cyclinD from forming complexes with cdk4/6. By sequestering cdk4/6, the cells are prevented from progressing through G₁. Cyclin E-cdk2 and cyclin A-cdk2 complexes, respectively, regulate late G₁ and early S phase. TGF β can also block the cell cycle progression mediated by cyclin E-cdk2 and cyclin A-cdk2 complexes indirectly via p15 by disrupting these complexes. The Cip/Kip family of cdk inhibitors, which include p21^{Cip/WAF1}, p27^{Kip1} and p57^{Kip2}, bind to these cyclin-cdk complexes causing their functional inactivation. CyclinD-cdk4/6 binds to p27 later in G₁ sequestering p27 and allowing cyclin E-cdk2 to become activated. TGF- β mediated induction of p15 allows its binding to the cyclin D-cdk4/6 complexes which frees p27, so that it can bind to cyclin E-cdk2 and prevent progression into S phase [174, 177, 178].

p21 is also known to bind to G₁ cdk, and recent reports suggest that p21 could also play an important effector role in TGF β growth suppression. While Smad3, Smad4 and Sp1 cooperate to activate the p21 promoter in HepG2 cells in response to TGF β , the DNA binding domain mutants of Smad3 and Smad4 as well as overexpression of Smad7 inhibited the induction of p21 by TGF β [92]. Despite general acceptance that p21 induces cell cycle arrest, clues to the molecular basis of its function have only recently started to emerge. HPV16 immortalized human ectocervical cells treated with TGF β were shown to have increased p53 levels that correlated with time and dose. In these cells, p21, bax and Mdm2 levels exhibited a concurrent increase with similar kinetics, while no distinguishable changes in the protein levels of cyclin D, cyclin E, cdk4, cdk6, cdk2, p27, p16 or RNA levels of p15 were observed. However, the catalytic activity of cdk2 was decreased, possibly by binding

to p21, which was shown to coimmunoprecipitate with cdk2 upon TGF β stimulation [179]. In gastric carcinoma cells, it has been reported that p21 inhibited the kinase activity of cyclin D and cyclin E associated cdk's resulting in hypophosphorylation of p130, a member of the RB family of proteins that regulate E2F [180]. Additionally, adenoviral E1A, a potent stimulator of cellular proliferation has also been shown to block TGF β induced induction of p15 and p21. The decrease in p15 appears to be at the level of transcriptional regulation and is dependent upon E1A's ability to bind p300 but independent of E1A's pRb binding activity [181]. Despite all these experimental data suggesting that TGF β growth inhibition is mediated *via* cdk inhibitors, it is also important to note that the growth inhibitory effects of TGF β are not solely mediated by cdk inhibitors as p15^{-/-} and p27^{-/-} mouse embryo fibroblasts are also sensitive to TGF β mediated growth inhibition [182, 183].

Downregulation of cdk activators is another mechanism by which TGF β can exert its growth suppressive effects. The cdc25A phosphatase removes tyrosine phosphorylation of cdk4 and cdk6, which in turn, activates these kinases. TGF β downregulates cdc25A, which prevents the removal of these inhibitory phosphate groups from and inhibits the catalytic activity of cdk4 and cdk6 [184]. The cdc25A downregulation involves a transcriptional repressor complex containing E2F, p130, and HDAC1, which bind to the promoter of cdc25A [1]. However, two studies have demonstrated that this downregulation of cdc25 is subsequent to p21 induction, which occurs very rapidly (< 1 hr) while the induction of p15 occurs at a later time point [184, 185].

Growth suppression by TGF β may also be activated upstream of cdk inhibitors by downregulating c-Myc. c-Myc is a ubiquitous promoter of cell growth and can act as both a transcriptional activator or repressor dependent upon the cofactors with which it associates [186]. The downregulation of c-Myc occurs very rapidly in many cells that are sensitive to TGF β growth inhibition [187]. The downregulation of c-Myc in response to TGF β has been proposed to be an essential early event prior to the induction of p15 and p21, as overexpression of c-Myc blocks p15 and p21 induction and ablates the cell cycle arrest [188, 189]. A positive association between c-Myc and cdc25A has been demonstrated and opens the possibility of another level of regulation of cdc25A by TGF β [190]. A genome wide analysis of rapid TGF β gene responses comparing human mammary epithelial cells with breast cancer cells demonstrated that c-Myc repression was selectively lost in the breast cancer cells. TGF β induces a Smad complex that binds to a TGF β inhibitory element in the c-Myc promoter. In the breast cancer cell line, these complexes were defective and the authors suggest that this Smad complex is a target of oncogenic signals in breast cancer [191]. Dual roles for the Myc-interacting zinc-finger protein 1 (Miz1) in response to TGF β signaling have recently been discovered, providing clues to the molecular basis of some of these end effects. Miz-1 relieves repression of p15^{INK4b} expression by interacting with Myc, preventing its recruitment to its promoter, and by enabling Smad protein complex mediated transactivation. [192].

While TGF β has also been shown to induce apoptosis in a number of cell types (hepatocytes, myeloid cells and

epithelial cells), organs and tissues, the mechanisms by which apoptosis is induced by TGF- β are poorly understood. TGF β induced apoptosis is essential for normal development of the neural crest, interdigital fields of the limb and the mammary gland ductal system [193-195]. There is also mounting indirect evidence implicating TGF β mediated apoptosis in the elimination of preneoplastic cells and that the abrogation of this pathway results in an increased rate of carcinogenesis and/or metastasis. The mechanisms regulating TGF β mediated apoptosis are just coming to light. There has been an increasing body of literature implicating Smad7 in the pathway. Smad7 expression is increased in rat prostatic epithelial cells undergoing apoptosis due to castration, and TGF β induced apoptosis was ablated by inhibiting Smad7 levels in a number of cell lines [196]. Smad7 expression increased apoptosis in Mv1Lu cells in response to TGF β and serum withdrawal. Furthermore, Smad7 decreased the activity of NF- κ B, a potent survival factor, and sensitized cells to various forms of cell death [197]. Smad3 and AP-1 may also play a role in this pathway as overexpression of Smad7 or dominant negative Smad3 was shown to block TGF β induced apoptosis [198]. Furthermore, the JunD-FosB-AP1 complex is also activated in TGF β dependent apoptosis. Dominant negative FosB inhibited apoptosis but not growth suppression in these cells [198]. These observations would predict that directly or indirectly disabling Smad3, Smad7 or other signaling molecules of the TGF β signaling pathway involved in apoptosis could facilitate tumor formation. Although gastrointestinal tumors have been observed in Smad3 knockout mice, so far there has been no evidence for mutational inactivation in human tumors suggesting that there might be indirect mechanisms for inactivation of this molecule in cancer.

Bcl and caspase family members have also been implicated in mediating the apoptotic pathway induced by TGF β [7, 199]. The NRP-154 rat prostate epithelial cell line can be induced to undergo apoptosis in response to TGF β . In this model, TGF β downregulates bcl-xL and PARP expression, promotes cytochrome c release and upregulates expression of caspases 3 and 9. Overexpression of bcl-xL prevented apoptosis by blocking cytochrome c release, activation of caspases 3 and 9 and cleavage of PARP. Interestingly, these cells were still sensitive to TGF β cell cycle arrest suggesting that there may be independent signaling events mediating these two processes [200]. A recent report suggests that bcl-xL downregulation is a result of reactive oxygen species (ROS) production by TGF β mediated effects. Decreased levels of bcl-xL cause a decrease in mitochondrial transmembrane potential, which may lead to the release of cytochrome c and the activation of caspase 3, resulting in apoptotic cell death [201]. Overall, these observations strongly imply that cell specific growth inhibitory or apoptotic responses mediated by TGF β *via* the Smads may become disabled in tumor cells enabling the uninhibited growth of tumors.

SMADS CROSSTALK WITH OTHER SIGNALING PATHWAYS

It is becoming increasingly clear that the TGF β signaling pathways are part of a signaling network that can lead to

numerous biological end effects. Signaling cascades involving Ras, JNKs, p38, and JAKs have been shown to interact with TGF β signaling at various levels leading to both synergistic and suppressive end results dependent upon the cellular context. MAP kinase pathways lead to the activation of either the ERK1/2, JNK or p38 kinase. The MAP kinase pathway is frequently dysregulated in cancer, most often due to oncogenic mutations in Ras, which has been shown to be an early event in a number of cancers [202-204]. Ras signaling has also been shown to be both antagonistic as well as cooperative with TGF β signaling. Ras antagonism can occur at various levels. Ras stimulates the activation of cyclin dependent kinases (CDKs) which are important in cellular progression through the cell cycle and hence proliferation. TGF β signaling induces the production of CDK inhibitors that can cancel the effects of CDK activation by Ras [177,205]. It has also been proposed that activation of the MAP kinase cascade by Ras can inactivate Smad signaling by sequestering Smad2 and Smad3 in the cytoplasm to such an extent that even overexpression of Smad4 is not able to restore TGF β signaling [206]. Additionally, oncogenic Ras signaling has been shown to increase the stability of the Smad co-repressor TGIF due to phosphorylation resulting in the suppression of expression of CDK inhibitors such as p15 [207]. Ras can also cooperate with TGF β signaling, and there is a growing body of evidence suggesting that Ras may "reroute" TGF β signaling in epithelial cells. Oncogenic Ras in mammary epithelial cells allows these cells to transdifferentiate into a highly invasive and metastatic phenotype while inhibiting Smad dependent growth inhibition [208]. Additionally, breast cancer cells with a constitutively activated Ras have an increased ability to metastasize to the bone in response to TGF β [209].

Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) have also been reported to activate Smad dependent gene transcription *via* an ERK dependent pathway in epithelial cells [210]. However, when phosphorylation of the linker region of Smad2 was catalyzed by MEKK1, it prevented the nuclear localization of Smad2 and thus Smad dependent transactivation [211]. Since phosphorylation of Smad2 usually occurs in the C-terminus of the protein in response to TGF β , it has been postulated that the sites where R-Smads are phosphorylated would ultimately determine whether growth inhibition or growth stimulation would occur during the signaling cascades.

TGF β can itself activate numerous MAP kinase pathways, including the MKK4-JNK and MKK3-p38 pathways that can lead to both Smad-dependent and Smad-independent transcription [85,212-215]. The activation of these cascades by TGF β may involve Rho-like guanosine triphosphatases [211]. TGF β activated kinase 1 (TAK1) may also be involved in TGF β signaling through p38 [216-218]. While TGF β activates the hematopoietic progenitor kinase (HPK) and an association between HPK and TAK1 has been shown, a direct link between TAK and TGF β RI is still unresolved [218]. TGF β activation of JNK or p38 can mediate transcription by activating AP-1 complexes *via* phosphorylation of c-Jun or CRE-regulatory complexes through ATF2 phosphorylation [85,213]. The specificity of these end effects may be mediated by the specific

interactions between Smads and ATF2 or Jun complexes that have recently been reported [85,87,216,219]. Interestingly, JNK has also been shown to phosphorylate Smads at undetermined sites within the linker region [220].

Finally, there is also emerging evidence to support crosstalk between TGF β signaling and the JAK/STAT and NF- κ B signaling pathways. Interferon- γ activates JAK tyrosine kinases which in turn activate STAT proteins. It has now been reported that Interferon- γ inhibits TGF β signaling by direct STAT-mediated transactivation of Smad7 [221]. Smad7 activation and inhibition of TGF β signaling has also been shown to be mediated by NF- κ B/RelA [222]. While all the details regarding the exact interactions that occur between TGF β signaling molecules and members of other signaling cascades remained unresolved, it is becoming increasingly clear that TGF β signaling is very complex with cell type and cellular micro- and macro-environment dictating which biological end results will occur.

TGF β SIGNALING IN ANGIOGENESIS AND METASTASIS

During early stages of tumor development, TGF β functions to suppress cell-cycle progression and inhibit tumor growth [173,177,223]. However, in later stages, human tumor cells generally develop resistance to TGF β -mediated growth inhibition [224]. In advanced cancers, TGF β ceases to function in tumor suppression and adopts the converse role of enhancing metastatic spread. Dissemination of malignant cells from a primary tumor to distal sites in the body is the principal cause of death in cancer patients. Metastasis can occur through a variety of mechanisms, including direct invasion of surrounding tissue (*per continuitatem*), dissemination *via* the blood vasculature (hematogenous metastasis), and/or through the lymphatic system (lymphatic metastasis) [225]. Overexpression of TGF β is detected in several advanced and metastatic human tumor types, including prostate, mammary, and renal cell cancers [226-229]. Elevated levels of TGF β are usually detected in the microenvironment surrounding the tumor and in the tumor stroma [230]. The excess TGF β may enhance tumor progression by promoting local tissue invasion and by inducing tumor angiogenesis. TGF β appears to play a role in both *per continuitatem* and hematogenous metastatic spread.

Metastasis *via* local tissue invasion is apparently promoted by TGF β signal transduction by altering the properties of the cell [208,223,231]. TGF β stimulates the conversion of fully polarized, non-invasive epithelial cells to an invasive mesenchymal, spindle cell-phenotype [231]. The epithelial to mesenchymal transition (EMT) involves decreased expression of genes important in cell-cell adhesion and increased expression of genes involved in cell-extracellular matrix connections [232]. The resulting cells display enhanced migration and invasion into surrounding tissues and demonstrate autocrine production of TGF β , which is essential to sustain the invasive property of the tumor cell [231]. On the contrary, interference with TGF β receptor signaling converts invasive, metastatic, mesenchymal cells to non-invasive cells with an epithelial phenotype [231]. Therefore, the TGF β receptors may be

promising targets for late-stage cancer therapeutics to revert invasive cells to a more benign phenotype.

Tumor angiogenesis, that is, the onset of neovascularization within a primary tumor, is essential for both tumor expansion and metastasis. The newfound access of the tumor to the host bloodstream not only affords necessary oxygen and nutrients to tumor cells, but also provides a route through which these cells may disseminate to distal sites [233]. The role of TGF β in blood vessel formation remains a highly complex process. TGF β exerts a biphasic effect on angiogenesis induced by vascular endothelial growth factor (VEGF). *In vitro*, high concentrations of TGF β inhibit endothelial invasion and capillary lumen formation, whereas lower concentrations of TGF β synergistically function to increase endothelial cell invasion mediated by VEGF or basic fibroblast growth factor (bFGF) [234, 235]. However, *in vivo*, TGF β has been shown to function in stimulating angiogenesis [236]. TGF β -1 knockout mice were found to suffer from defective vasculogenesis and embryonic lethality, supporting an essential role for TGF β signaling in blood vessel formation [237].

The activin receptor-like kinases (ALKs) are TGF β RI receptors, which seem to play a role in blood vessel formation and remodeling. ALK1 and ALK5 induce different TGF β signaling pathways that determine the phenotype of the endothelium during angiogenesis; endothelial cells alternate between an activation phase and a resolution phase during blood vessel formation [238]. TGF β signaling through ALK5 seems to promote passage into the activation phase, which is characterized by migration and proliferation of endothelial cells to generate vessel formation. However, signaling through ALK1 appears to induce the resolution phase in which endothelial cells cease migration and proliferation and the basement membrane becomes reconstituted [239]. It has been speculated that ALK5 has a higher sensitivity to TGF β than ALK1; low concentrations of TGF β would therefore only activate ALK5 and not ALK1, thus favoring endothelial cell proliferation. Higher concentrations of TGF β would bind and activate ALK1, which inhibits the ALK5 pathway and thus concludes the process of angiogenesis [239]. As demonstrated in the aforementioned *in vitro* studies, varying concentrations of TGF β apparently affect angiogenic activity *via* the mediation of VEGF and bFGF; it can be hypothesized that TGF β signaling through ALK1 and ALK5 regulates angiogenesis in a similar manner. However, further studies are required to elucidate the exact nature of the signaling events mediated by ALK1 and ALK5.

The recruitment of excess TGF β to areas of angiogenic activity may be regulated by CD105 (endoglin). This glycoprotein is similar to the RIII receptor for TGF β , and is expressed predominantly in proliferating endothelial cells. Expression levels of CD105 are particularly elevated in endothelial cells of tumor blood vessels, but are virtually undetectable in blood vessels of normal tissue [240-242]. Overexpression of CD105 has been linked to blood vessel formation, whereas CD105 null mice are severely defective in angiogenesis and die *in utero* [243]. Thus, expression of CD105 may mediate the cellular proliferation effects of TGF β in the blood vessel endothelium, promoting angiogenesis and subsequently metastasis.

Due to the limited availability of data, the overall role of TGF β signaling in stimulating angiogenesis appears highly complex and is largely unresolved. However, a recent report demonstrates that Smad4 inhibits expression of VEGF, a potent inducer of angiogenesis, and increases expression of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis [244]. Hence, inactivation of Smad4, as observed at high frequency in colorectal and pancreatic cancers, would implicate promotion of tumor angiogenesis and metastasis. This notion has been further supported by recent experimental data from the analysis of human colorectal cancer where a strong correlation between higher frequency of Smad4 gene mutations and distant metastases relative to non-metastatic forms of colon cancer was observed [154]. This data is also consistent with a role for Smad4 inactivation in the stimulation of angiogenesis, which could in turn permit hematogenous metastasis. However, it seems highly likely that the function of TGF β in tumor blood vessel formation is reliant upon both the tumor type and the particular mutation in the TGF β signaling pathway.

TGF β is also known to induce the expression of various components of the extracellular matrix (ECM), including collagen, fibronectin, tenascin, vitronectin, proteoglycans and integrins. In some cell types, this growth factor also functions to inhibit degradation of the matrix by repressing expression of proteases, including plasminogen activators, collagenase and stromelysin and through activating protease inhibitors, such as TIMP1 and PAI-1 [245-248]. Degradation and remodeling of the ECM are key events in both angiogenesis and metastasis. In order to disseminate, tumor cells must detach from neighboring cells and escape from the tissue of origin *via* either hematogenous or lymphatic routes and hence the breakdown of the ECM would hasten the flight of the tumor cells to distal sites [249]. Thus, blood vessel and possibly lymphatic vessel formation is facilitated by degradation of the ECM. The breakdown of connective tissue barriers creates a path through which endothelial cells are able to migrate, adhere and proliferate, generating new vessels which may grow toward and infiltrate the tumor mass.

Despite a plethora of evidence presented in the literature for promotion of ECM formation by TGF β , contradictory roles for TGF β in the maintenance of the ECM have also been observed in the tumorigenic process. In malignant cells that have acquired TGF β -resistance, one may expect that pathways that induce expression of ECM molecules would not be intact. However, TGF β retains its ability to induce ECM as well as to positively stimulate breakdown of the ECM through induction of type IV collagenases. These matrix metalloproteases are involved in the degradation of basement membrane and promote metastatic characteristics, which are often observed in studies using tumor cell lines as the experimental system [250-253]. The role of TGF β in ECM maintenance is primarily mediated through RI receptor signaling [254]. However, it has been postulated that TGF β regulates certain ECM molecules independently, possibly through different receptor subtypes or alternative signaling molecules [248]. In contrast to the degradation of ECM promoted by TGF β during tumor metastasis, other observations show an inhibition of ECM degradation by TGF β -mediated stimulation of the synthesis of PAI-1 in both

primary and metastatic tumors [248]. Therefore, the role of TGF β in promoting ECM degradation, and thus allowing metastasis, is not globally applicable to all cancers and may be dependent on the specific mutated targets in the TGF β signaling pathways.

Recent studies have provided additional evidence to confirm that TGF β -mediated growth suppression and apoptosis may operate independently of the angiogenic and metastatic processes. Activated Ha-Ras collaborates with TGF β to induce the phenotypic invasiveness of mammary epithelial tumor cells, whereas Raf-induced TGF β production blocks its apoptotic but not its invasive responses in epithelial cells [231, 255]. Additionally, work using breast cancer cell lines has shown a requirement for Smad2 and Smad4 in inducing trans-differentiation in cells with low levels of RI receptors [256]. Furthermore, a recent study reports that blocking TGF- β signaling with truncated RII receptors has no effect on the local growth of the tumor, but significantly decreases metastasis to the bone [209].

Although there is increasing evidence for TGF β involvement in metastasis through both local tissue invasion and hematogenous spread, it remains to be determined whether TGF β plays a role in lymphatic metastasis. Tumor cell dissemination *via* the lymphatic system has received secondary consideration relative to tumor metastasis *via* local tissue invasion or *via* the blood vascular system. With the recent cloning of the lymphatic vessel specific growth factors, VEGF-C and VEGF-D, focus has shifted toward the lymphatic system as an important medium for metastasis [257-259]. These observations prompt one to ask whether a relationship exists between TGF β signaling and lymphatic metastasis, or more specifically, between TGF β and VEGF-C and/or VEGF-D. A refined understanding of the molecular mechanisms underlying metastasis will elucidate the contribution of TGF β signaling to tumor cell dissemination.

TGF β AND IMMUNE SUPPRESSION

The critical roles of TGF β in regulating the immune system have recently received increasing attention. TGF β is a multi-functional cytokine with a number of roles in the immune system [4, 260]. Many immune cells, including lymphocytes, macrophages and dendritic cells, produce TGF β ; this expression is regulated by both autocrine and paracrine pathways, and TGF β mediates the differentiation, selection, apoptosis, activation and proliferation of these immune cells [4, 261]. The characterization of the Smad family of proteins has helped to elucidate the molecular mechanisms underlying the effects of TGF β in the immune system, including cross talk with other cytokine and lymphokine pathways [221, 262].

T lymphocytes are regulated by TGF β at all stages of development, from differentiation to activation and proliferation [263, 264]. Some subsets of activated T cells (Th3 cells) are able to synthesize and secrete TGF β , which typically inhibits production of interleukin-2 (IL-2), thus suppressing T cell proliferation. Consistent with these observations, TGF β 1 null mice exhibit an increased expression of IL-2, class II MHC antigen and primary

expansion of CD4 $^{+}$ T cells [265, 266]. Such mice also develop a multifocal inflammatory disease with autoimmune manifestations, including production of autoantibodies [267]. During T cell education, TGF β may regulate maturation of double positive T cells from CD4 $^{+}$ CD8 low precursors; in the absence of TGF β 1, double positive thymocytes are generated too rapidly to allow appropriate selection processes to transpire. Dysregulated production of CD4 $^{+}$ CD8 $^{+}$ T cells in these mice may be exacerbated by defects in apoptosis of T cell subsets, thus causing autoimmune disease [263].

Cytotoxic T cells (CTLs), or class I MHC-restricted T cells, can provide an effective anti-tumor defense in cell-mediated immune responses. These effector cells are responsible for direct killing of virus-infected or allogeneic cells [268]. CTLs exert their effect by lysing cells through one of two mechanisms: the perforin/granzyme B pathway or the Fas/Fas ligand pathway. TGF β 1 plays a role in determining cytotoxicity by regulating the repertoire of gene expression in the CTLs. Several studies have demonstrated *in vitro* that TGF β 1 inhibits perforin mRNA expression and thus suppresses the perforin/granzyme B cytotoxic pathway; however, TGF β 1 has no effect on the Fas/FasL cytotoxic pathways [269, 270]. TGF β 1 may also decrease immunosurveillance by CTLs in a more indirect manner through suppressing expression of helper T cells. CD4 $^{+}$ helper T cells (Th cells), or class II MHC restricted T cells, are not generally cytotoxic to tumors; however, these cells may play a role in the anti-tumor response by providing cytokines for the effective development of CTLs [268]. Recent studies have shown that TGF β 1 suppresses memory Th1 T cell maturation by down-regulating expression of the β 2 chain of the IL-12 receptor; IL-12 is required for a Th1 cell to develop from a naïve CD4 $^{+}$ T cell. TGF β 1 also inhibits the development of Th2 cells. During cancer progression, TGF β 1 suppression of the expression of perforin and prevention of the maturation of Th1 and Th2 cells may thus inhibit anti-tumor immune responses.

It remains difficult to develop a comprehensive view of the effect of TGF β on T lymphocytes as TGF β works not only directly but also indirectly on T cells by affecting antigen-presenting cells (APCs). Dendritic cells (DCs) are typical APCs in both lymphoid and non-lymphoid organs; they function to present peptide antigens to helper T cells and to initiate antigen-specific T cell proliferation. Several studies have reported that TGF β inhibits *in vitro* activation and maturation of lymphoid tissue type DCs [271]. TGF β may inhibit regulation of T cell costimulatory molecules on the surface of these DCs and thus reduce their antigen presenting capacity. The immunosuppressive role of TGF β in cellular immunology is therefore due to its inhibitory effect on both effector T cells and APCs.

TGF β may also mediate several aspects of the maturation and differentiation functions of B cells. *In vitro*, TGF β can inhibit B cell proliferation, antibody secretion and expression of surface molecules, including antigen receptors. In humans, TGF β is also involved in directing switch recombination in immunoglobulin isotype IgA; in TGF β null mice, high levels of autoantibodies, predominantly IgG, are detected [272, 273]. Several human and murine B lymphoid malignancies have been identified in which B cells become

resistant to the growth inhibitory effects of TGF β ; these malignant B cells express substantial amounts of active TGF β . These insensitivities may be derived from defects in any of the players of the TGF β signaling pathway, including the Smads. Further studies are necessary to determine the molecular basis of these defects.

Studies of pathogens suggest that increased production of TGF β may lead to decreased immune surveillance. The immune response to a variety of pathogens, including viruses, bacteria, yeast and protozoa, is regulated by TGF β . *Trypanosoma cruzi*, a protozoan parasite, infects macrophages and suppresses their antibacterial activity; these macrophages are stimulated by the parasite to produce increased levels of TGF β [274]. Studies, which have focused on mycobacteria and viruses, including the human immunodeficiency virus (HIV), have reported that TGF β has both a negative influence on host immune response and a beneficial effect on the survival and growth of intracellular pathogens [275].

TGF β may allow tumor progression through its ability to suppress immune surveillance. An increase in the expression of TGF β is common in carcinomas resistant to TGF β -mediated growth inhibition and is also apparently important for tumor progression; this association may also play a role in suppression of immune surveillance [276-278]. Expression of TGF β allows disseminating tumor cells to permeate the body without being attacked by mediators of the immune response such as cytotoxic T cells and macrophages. In some primary tumor cells, increased expression of TGF β and interleukin 10 (IL-10), another potent immunosuppressive factor, has been detected; expression of such genes may explain how tumors suppress CTL function and escape from the immune response [279, 280]. The analysis of genes expressed by tumor cells using high throughput methods may provide the critical data required to discover which other cytokines or factors are involved in the effective immune suppression achieved by tumors [281-283].

GENE KNOCKOUT STUDIES

TGF β expression begins early in development, and gene targeting approaches have been used to elucidate the *in vivo*

functions of both TGF β isoforms and Smads. TGF β 1 (-/-) mice develop a progressive wasting syndrome resulting in death within a few days [254, 255]. Detailed analysis showed infiltration of lymphocytes and macrophages and necrosis in many of the organs, especially the heart and lungs [254-257]. Many of the lesions found in these mice resembled those found in autoimmune disorders, suggesting a role for TGF β 1 in immune cell proliferation as we have already described in detail in the previous section of this review [235, 258]. An embryonic phenotype has also been observed in the TGF β 1 (-/-) mice as only 50% of the conceptuses reach parturition [207, 254, 255]. These mice die by E10.5 due to defective yolk vasculogenesis as well as defective hematopoiesis [207]. However, the TGF β 1 (-/-) mice, when derived from a 129 background, develop nonmetastatic colon cancer if they are rescued from autoimmune disease [259, 260]. Additionally, TGF β 1 may also play a role in susceptibility to carcinogenesis. TGF β 1 (+/-) mice have an enhanced propensity to develop lung and liver cancer when treated with carcinogens.

TGF β 2 (-/-) mice die from congenital cyanosis shortly before or during birth. They have profound craniofacial, eye, spinal column, cardiac, lung, limb and urogenital defects [261]. TGF β 3 (-/-) mice die within 24 hrs of birth. They have cleft palates and abnormal pulmonary histology, but most of the remaining organs are normal [262, 263].

TGF β receptor RII knockouts result in embryonic lethality. Null mice die at E10.5 due to defects in yolk sac hematopoiesis and vasculogenesis [264]. These mice resemble the TGF β 1 (-/-) mice suggesting that the TGF β -receptor RII may play a role in hematopoiesis and endothelial differentiation.

Smad knockout studies have revealed that all of the Smad knockouts exhibit developmental defects (Table 5). While the Smad 2, 4 and 5 knockouts are embryonic lethal, the Smad 3 and 6 knockout mice survive to term. Smad 2 (-/-) mice die before E8.5 [265-268]. These Smad2 null embryos do not form a head fold or primitive streak, lack the extraembryonic portion of the egg cylinder, have no mesoderm formation and do not undergo gastrulation. Smad 3 null mice are born viable and fertile [269, 270]. However, these mice die between one and eight months of age due to

Table 5. Smad Knockout Mice

Gene	-/- Phenotype	References
Smad2	Embryonic lethality by E7.5-E8.5. Defects in egg cylinder elongation, mesoderm formation, and gastrulation. Anterior-posterior axis formation abnormalities.	[295-298]
Smad3	Death within 1-10 months. Metastatic colorectal cancer at 4-6 months of age. Immune dysregulation, severe mucosal infection, accelerated wound healing, osteoporosis and skeletal defects.	[299-301]
Smad4	Embryonic lethality by E7.5-E8.5. Growth retardation. Abnormal visceral endoderm formation, absence of mesoderm and lack of gastrulation. Primary defects in extraembryonic tissue development and abnormalities in anterior-posterior axis formation.	[302, 303, 304]
Smad5	Embryonic lethality by E9.5-E10.5. Defects in angiogenesis, mesenchymal apoptosis and in gut, heart and craniofacial development.	[306-308]
Smad6	Cardiovascular abnormalities and defects in endocardial cushion transformation.	[309]

chronic infection, suggesting that Smad 3 is a player in immune responses [269, 271]. Fibroblasts from the Smad 3 null mice have been shown to be partially resistant to TGF β dependent growth inhibition [269]. In some genetic backgrounds, Smad3 (-/-) homozygotes develop colorectal tumors ranging from hyperplastic lesions and polyps to highly aggressive tumors, which are very invasive and highly metastatic, with dissemination to the lymph nodes [270]. The Smad 4 null phenotype is similar to that of the Smad 2 knockout. Smad 4 (-/-) mice die before E7.5, are severely growth retarded, fail to gastrulate, fail to form the egg cylinder, and show abnormal visceral endoderm development [272]. It was originally reported that Smad 4 heterozygous mice show no increase in tumorigenicity [272]. However, a recent report has shown that Smad4 heterozygous mice develop gastric polyps that can develop into tumors at a late age [273]. Furthermore, a compound heterozygote mouse carrying mutations in the *APC* (adenomatous polyposis coli) gene (Δ 716) and Smad4 exhibited intestinal polyps which developed into malignant tumors to a greater extent than those in the simple *APC* heterozygotes, suggesting that mutations in Smad4 play a significant role in the malignant progression of colorectal tumors [274]. Smad 5 (-/-) mice die at E9.5 – E11.5, and have numerous defects in angiogenesis, including enlarged vessels and fewer smooth muscle cells. Smad5 (-/-) homozygotes have left/right asymmetry, craniofacial abnormalities, and they undergo extensive mesenchymal apoptosis [275-277]. Smad 6 knockouts are viable, but have a number of cardiac abnormalities, suggesting a role for Smad 6 in the development and homeostasis of the cardiac system. These Smad 6 (-/-) mice have hyperplasia of the cardiac valves, outflow tract septation defects, aortic ossification and elevated blood pressure [278].

FUTURE PERSPECTIVES

The TGF β signaling pathways have gained importance in cancer research due to their opposing roles in both tumor suppression and metastatic cancer promotion elicited *via* specific end effects. Many researchers have reported conflicting evidence when undertaking very similar experiments. How can some groups detect growth inhibitory effects, and hence resistance to tumorigenesis, while others report invasiveness and metastasis? Often times, cell type specificity may be explained by a unique set of constitutively expressed and/or inducible protein factors which affects the internal cellular environment. However, in the case of TGF β , the answer is much more complex due to the influence of the local and/or overall environment of the target tissue. TGF β signaling can be affected by externally introduced agents, as demonstrated in studies showing that smoking causes a further increase in the plasma levels of TGF β in patients with diabetes, and that exposure to hydrogen peroxide causes the induction of TGF β 1 and an associated increase in ECM components [310, 311]. The identity and roles of the various chemicals or other agents introduced from the environment, which affect TGF β signaling and hence influence the development or metastatic spread of tumors, is still awaiting further research. These exogenous factors may cause specific genetic or epigenetic alterations at certain hot spots ultimately leading to localized deletions such as that of

chromosome 18q and thus inactivation of target genes [11,23,24,142,312,313]. Alternatively, these factors may cause non-genetic alterations of the functional outcomes of the various signaling events, such as the rerouting of standard signaling pathways. The end results of these changes that may contribute to cancer could be comprised of, but are not limited to, the prevention of cellular turnover due to ablation of normal TGF β growth inhibition, inhibition of apoptosis, increase in malignant conversion of cells affording angiogenesis, tumor invasion and metastasis and enhancement of the events that normally disable immunosuppressive effects.

Although the molecular basis of TGF β signaling events are becoming increasingly clear due to the discovery of critical signaling mediators such as the Smad proteins, much work remains for the research community. Of importance is the need to unravel the end effects resulting from the various combinatorial signaling events at the level of each signaling event, the signaling cross talk and the environmental effects that may ultimately be responsible for the development of cancer. Additionally, there is an emerging general acceptance that human TGF β signaling is mediated by Smads, involving different R-Smads and the only known Co-Smad, Smad4. However, recent studies might suggest otherwise due to the evidence for TGF β mediated growth inhibition in Smad4 disabled cells and due to the demonstration of the existence of Smad4 homologues in other vertebrates [46, 314]. These observations strongly suggest that there might be other TGF β signaling pathways operative in the cells, which involve other Smad4 homologues or Smad4 independent events. The rapid development of high throughput genomic methodologies and data produced from the application of these methods is expected to aid in understanding these complexities and in providing new leads for delineating TGF β -mediated signaling events. The availability of human sequence data can be taken advantage of to speed up the discovery of novel genes as well as the discovery of new members of the *Smad*, *TGF β* ligand and *TGF β* receptor families [315, 316]. Furthermore, the systematic use of high throughput methods such as gene expression microarrays, SAGE (serial analysis of gene expression) and proteomics in conjunction with gene knockouts in cell lines and animal models may ultimately help in determining the various players in the signaling events as well as in eliciting their biological end effects [281-283]. These studies would provide an overall picture of TGF β signaling and help to determine the critical steps or target genes in which inactivations may lead to cancer, thus aiding in the identification of nodal points for targeting therapeutic applications to contain and combat cancer.

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Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence

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High frequency of chromosomal deletions elicited as losses of heterozygosity is a hallmark of genomic instability in cancer. Functional losses of tumor suppressor genes caused by loss of heterozygosity at defined regions during clonal selection for growth advantage define the minimally lost regions as their likely locations on chromosomes. Loss of heterozygosity is elicited at the molecular or cytogenetic level as a deletion, a gene conversion, single or double homologous and nonhomologous mitotic recombinations, a translocation, chromosome breakage and loss, chromosomal fusion or telomeric end-to-end fusions, or whole chromosome loss with or without accompanying duplication of the retained chromosome. Because of the high level of specificity, loss of heterozygosity has recently become invaluable as a marker for diagnosis and prognosis of cancer. The molecular defects for the occurrence of loss of heterozygosity are derived from disabled caretaker genes, which protect the integrity of DNA, or chromosome segregator genes, which mediate faithful chromosome disjunction. *Curr Opin Oncol* 2002, 14:65–72 © 2002

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Abbreviations

DSB	double-strand break
HEJ	homologous end joining
LOH	loss of heterozygosity
MLR	minimally lost region
NHEJ	nonhomologous end joining
TSG	tumor suppressor gene

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Genetic alterations and cancer

Human cancer is generally thought to develop as a result of loss of or irreversible damage to critical genes in a multistep process involving the accumulation of genetic alterations. More than 100 years ago, Theodor Boveri wrote a remarkable book, *The Origins of Malignant Tumors* [1,2], in which he suggested chromosome missegregation leading to aneuploidy, an abnormal balance of chromosomes, as the fundamental basis of cancer. The aneuploidy hypothesis has since been abandoned in favor of the gene mutation hypothesis [3,4••]. However, selection of nonparental aggressive karyotypic variants of cancer cells caused by genetic instability and mutations could be unified in aneuploidy to explain tumorigenesis [5–7,8••].

Overall, the genesis of cancer could be defined as the manifestation of loss of or abnormal function of genes affecting processes that maintain or regulate orderly normal cell function by both genetic and epigenetic mechanisms. The genetic basis of these functional anomalies could be derived from targeted aberrations in the regulatory elements or functional domains because of mutations or loss caused by deletions of small or large contiguous genetic material affecting nonessential but critical genes required for normal differentiated function. During localized evolution, tumor cells aggressively proliferate and invade and spread to distant sites. At the same time, the genetic material could undergo accompanying changes, potentially in small increments, to aid in clonal evolution, increasing the genetic heterogeneity of the tumor cells. Therefore, the genetic outlook and the biochemical properties of the tumor cells at the time of initiation, could be entirely different from their characteristics at an advanced stage of cancer. These abnormalities in the blueprint of the genetic material could be derived from defective oncogenes, which become activated because of alteration of one allele, or from tumor suppressor genes (TSGs), whose functionality is eliminated when both alleles are damaged or lost.

Loss of heterozygosity and mapping of tumor suppressor genes in cancer

A statistical study of a childhood cancer, retinoblastoma, led to the proposal of the two-mutation hypothesis for the initiation of cancer, in which the first mutation could

be either germinal or somatic and the second was always somatic [9,10,11••]. This hypothesis was experimentally confirmed in subsequent studies by the demonstration of loss of heterozygosity (LOH) at the *RB1* locus in retinoblastoma patients carrying a germ line mutation of the *RB1* gene [12,13]. LOH, defined as a loss of one allele at a constitutional (germline) heterozygous locus, has been accepted as a hallmark of one of the two hits required for the inactivation of TSGs in cancer.

Loss of heterozygosity analyses of solid tumors have not only enabled the delineation of specific minimally lost regions (MLRs) as the likely locations of critical TSGs but also provided the molecular portrait of the pattern of accumulation of genetic alterations in a multistep progression of cancer [14–18,19••]. Confirmed TSGs could have been isolated by either linkage studies or LOH analyses of sporadic tumors, because the MLRs almost always point to the map position of critical TSGs involved in different types of cancers [19••,20–30,31•]. A TSG involved in a sporadic cancer could be a familial gene for a different cancer, and *vice versa* (Table 1).

A plethora of evidence supporting sites of recurrent LOH, which underlies the growth advantage required for tumorigenesis, still has not resolved the question of whether the mutation or LOH occurs first to initiate cancer. Localized double deletions (homozygous) targeting a specific gene or locus are rare. LOH patterns and the extent of each deletion in clonally selected tumor cells are highly specific to the chromosome [19••]. These discrepancies apparently reflect the size of the chromosome (*ie*, smaller chromosomes exhibit whole chromosome losses more frequently, whereas larger ones predominantly harbor partial losses) and selective pressure imposed by the remaining genes, which are essential for cell survival or provide a growth advantage during clonal selection of cells that eventually form the tumor. Overall, when one of the hits is LOH encompassing the entire chromosome, a portion, or a localized region, the second hit is inactivation of the actual target gene caused by a second reciprocal LOH event (homozygous), a mu-

tation, or an epigenetic alteration resulting in loss of gene function.

Despite the confirmation of two-hit inactivation of TSGs in several cancers, the mode of inactivation itself may vary in a gene-specific or tumor-specific manner. Two of the most historically celebrated TSGs are *Rb1* and *p53*. Retinoblastoma and its associated tumors, such as osteosarcoma, were found to harbor consistently either a homozygous deletion of the *Rb1* locus at 13q14 or a mutation of the remaining allele in a tumor with LOH [17]. A consistent region of LOH at 17p13, observed initially in colon cancer, led to the rediscovery of the *p53* gene as a TSG [32,33]. Missense mutations were the primary mechanism of inactivation of the remaining allele, and *p53* was later confirmed as the most frequent target for inactivation in a variety of cancers [32–34]. These observations are consistent with mutational inactivation of the target gene followed by LOH in familial cancers (*eg*, retinoblastoma, Li-Fraumeni) as the predominant mechanism caused by a predisposing genetic alteration, whereas in sporadic cancers, the mode of inactivation of the target gene may be variable, but the ultimate outcome is the loss of function. Exceptions to these rules have been observed in several cancers, and whether mutation or LOH occurs first in target inactivation is being debated [35••,36••,37•]. Although this debate is primarily based on studies of sporadic cancers in which a normal or mutant allele of the suspected target TSG has been observed in tumors exhibiting LOH, the explanation of these assessments could be more complicated because of the potential presence of multiple target genes, alternate modes of inactivation such as epigenetic silencing of gene expression by promoter methylation, or linkage disequilibrium with the true target gene.

The inactivation of the *Smad2* and *Smad4* genes, localized to the MLR at 18q21 in colorectal cancer, is consistent with the multiple target gene theory, in which true inactivation of both genes has been demonstrated [25,38]. The *p16* (*CDKN2A*) gene localizes to chromosome 9p21, also a hot spot for inactivation by LOH. Al-

Table 1. Tumor suppressor genes and cancers

Gene	Map position	Familial cancer syndromes	Associated sporadic cancers
<i>APC</i>	5q21	Adenomatous polyposis coli and Turcot syndrome	Colon and brain cancers
<i>BRCA1</i>	17q21	Breast/ovarian cancer	Breast, ovarian, and prostate cancer
<i>BRCA2</i>	13q12–13	Breast cancer	Breast, ovarian, and pancreatic cancers
<i>NF1</i>	17q11.2	Neurofibromatosis type I	Neurofibromatosis, colon carcinoma, and astrocytoma
<i>NF2</i>	22q12.2	Neurofibromatosis type II	Vestibular schwannoma, meningioma, and ependymoma
<i>p16^{MTS1}</i>	9p21	Melanoma	Glioblastoma, melanoma, and cancers of the pancreas, breast, and other organs
<i>p53</i>	17p13	Li-Fraumeni syndrome	50% of all cancers, including breast, brain, lung, colon, bladder, ovarian, and prostate
<i>RB1</i>	13q14.3	Retinoblastoma	Retinoblastoma, osteosarcoma, bladder carcinoma, and cancers of the breast and lung
<i>SMAD4</i>	18q21	Juvenile polyposis	Pancreatic, colon, and lung cancers
<i>PTEN</i>	10q24–25	Cowden disease	Brain, breast, and prostate cancers
<i>VHL</i>	3p25–26	von Hippel-Lindau syndrome	Renal cell carcinoma and pheochromocytoma
<i>WT1</i>	11p13–15	Wilm tumor	Nephroblastoma

terations in *p16* occur frequently in lung, skin, and most other common forms of human cancer [39,40]. In many tumor types, point mutations in the *p16* gene are rare, and LOH of 9p21 is not always observed in tumors. However, there is increasing evidence for loss of *p16* gene function via transcriptional silencing associated with abnormal DNA methylation of the transcription regulatory region [41]. Allele-specific methylation at particular sites may be somatic or may occur in the germ line, affecting all cells. Therefore, the two-hit hypothesis of TSG inactivation by Knudson [9–11] could be extended to explain that the first hit, the second hit, or both could result from methylation, leading to complete inactivation of the gene [42,43••]. The exploitation of the known modes of inactivation of the TSGs to tumors harboring LOH of a specific region may not be sufficient to account completely for the two hits required for the inactivation in all tumors. This situation has been frequently encountered with several TSGs, including *p16* and *PTEN*, during the analyses of melanomas and glioblastomas, and even with *p53* in some cases of Li-Fraumeni, implying that these suggested target genes may be in linkage disequilibrium with the true target gene or may be inactivated at a different point of the functional pathway controlled by these genes [44,45••,

46,47••,48,49]. Delineation of all candidate genes localized to the MLRs and painstaking evaluations to determine their loss of function unambiguously would be necessary to answer these questions. Assignment of true target genes is a tedious process but could be expedited by technologic advances in analyzing loss of function of genes and improvements in making accurate genome sequence data available in a short time using a high throughput method.

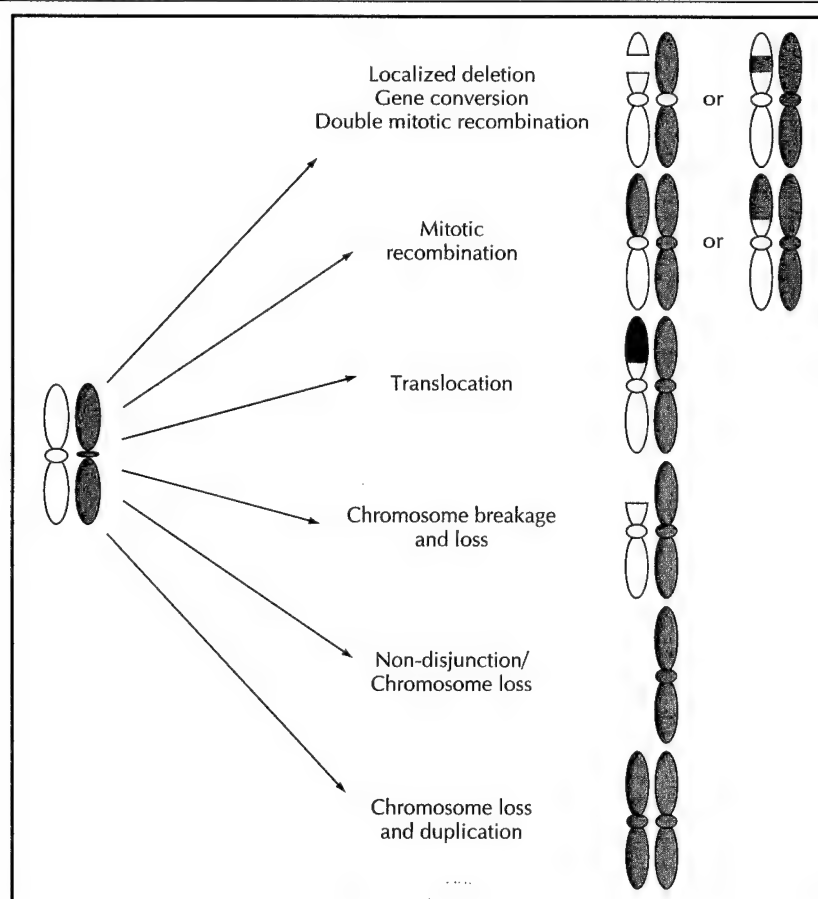
Identification of target genes is actively pursued on one front, but utilization of available LOH data as markers for diagnosis and prognosis of cancer also has become generally accepted. A higher frequency of consistent LOH at defined chromosomal regions critical for specific cancers has made this a useful, reliable DNA marker for diagnosis and prognosis of cancer, regardless of whether the target gene has been identified [50–53].

Molecular elicitation of loss of heterozygosity

A heuristic model summarizing the molecular elicitation of LOH in a variety of tumors is presented in Figure 1. Variations in the patterns in which LOH could occur include the following:

Figure 1. A model for molecular elicitation of loss of heterozygosity

Events such as localized deletions with accompanying chromatin loss resulting from one or two double-strand DNA breaks, gene conversion, single or double mitotic recombination, translocation, chromosomal nondisjunction, and chromosomal nondisjunction accompanied by duplication of the remaining chromosome could lead to loss of heterozygosity (LOH). The loss of genes localized to the regions of LOH could result in haploinsufficiency or unmasking of the functional expression of a recessive or deficient allele.



(1) Localized loss of one allele or gene in a highly specific manner could be accomplished by a simple deletion resulting from two double-strand breaks or double mitotic recombination involving the homologous chromosomal arm. When this occurs, the unaffected genetic material remains contiguous. If the loss involved a specific gene, it can be regarded as a gene conversion. Allele-specific or gene-specific probes and flanking probes would be necessary to detect such alterations. Although these microdeletions are difficult and laborious to find because of the enormous amount of effort required to analyze numerous tumor samples, at least two recent reports, one analyzing the *NF1* locus on 17q in neurofibromas and the other delineating a highly specific localized LOH on chromosome 1p for colon cancers, substantiate the claim that interstitial deletions targeting a single gene or allele could occur [19••,54••]. Additionally, highly specific, localized homozygous deletions observed within a gene or locus also indicate that a double hit could occur simply by targeted loss of genetic material [25,55–57].

(2) Extensive loss of genetic material involving a portion of or an entire chromosomal arm could be accomplished by a double-strand break with the loss of genetic material distal to the break, a single mitotic recombination involving the homologous pair of chromosomes, or reciprocal or nonreciprocal translocation. Mitotic recombination is the result of a reciprocal exchange of genetic material between nonsister chromatids of homologous or nonhomologous chromosomes in mitotic cells as detected by substitution of contiguous markers with reference to an established marker. Although these are the most common genetic abnormalities described for tumor cells, in a recent landmark study, the authors confirmed the previous observations and suggestions and provided comparative and direct molecular genetic and cytogenetic evi-

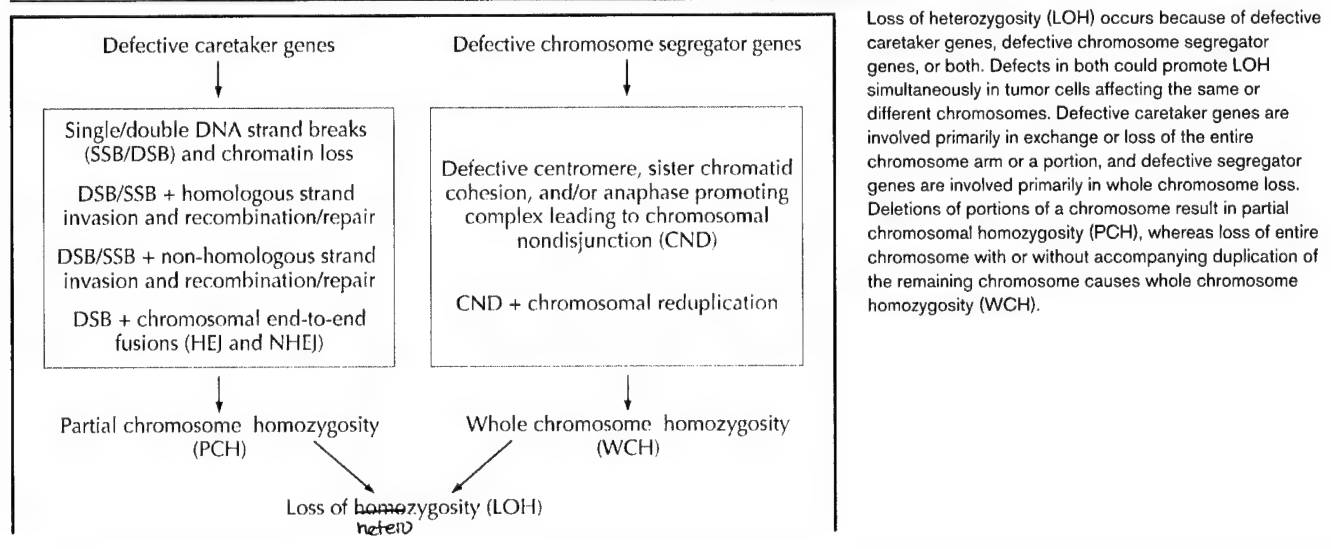
dence from the same tumor-derived cells for the existence of isochromosomes, translocations, and complete loss of genetic material to support additional mechanisms for LOH [12,14,19••,25,30,57–61].

(3) Loss of a whole chromosome is generally accomplished by nondisjunction defects in chromosome segregation [26,32]. However, the existence of multiple copies of chromosomes in tumor cells deemed to have lost one member of the homologous pair by cytogenetic analyses confirms reduplication of the remaining chromosome leading to homozygosity [19••,59,62,63].

Roles of caretaker and chromosome segregator genes in loss of heterozygosity

Multiple interconnected mechanisms have evolved to ensure the maintenance and faithful partitioning of genetic material at cell division. The failure of cellular functions to maintain the genetic integrity of the genome by faithful DNA replication, DNA damage repair, telomere protection, segregation of chromosomes at mitosis, or unscheduled recombination could lead to genomic instability, which is elicited as LOH. The two major categories of chromosomal abnormalities that involve portions of a chromosome or an entire chromosome could be considered derived from defects in caretaker genes or chromosome segregator genes, respectively (Fig. 2). Broadly, a caretaker gene is any gene required to maintain the integrity of DNA during processes such as DNA replication, repair, or recombination; telomere maintenance and protection; and chromosome packaging; or, to protect the DNA from nucleases and other adversities of intracellular physiologic byproducts [64–66,67•,68•,69••]. The chromosome segregator genes are those required to mediate orderly disjunction of sister chromatids to the daughter cells during a mitotic cell division. These genes could include all those involved in determining the cen-

Figure 2. A model for the genetic and molecular mechanistic basis of loss of heterozygosity



tromere structure, sister chromatid cohesion, and the anaphase promoting complex, and others required for the proper assembly of the bipolar spindle apparatus to ensure faithful segregation of genetic information [70,71,72••,73,74•].

Single-strand DNA interruptions, breaks, or gaps could arise during DNA damage from a variety of causes, such as exposure to ultraviolet radiation, hydrogen peroxide or alkylating agents, DNA replication accompanied by processes such as defective nucleotide excision repair or mismatch repair, or defective DNA replication resulting in the accumulation of the lagging strand caused by defective DNA ligase [65,75–78]. Single-strand DNA generated during these events could enhance mitotic recombination and lead to an exchange of genetic material from homologous or nonhomologous chromosomes. A number of genetically determined disorders are known to cause susceptibility to chromosomal breaks, cause increased frequency of breaks, and promote interchanges that occur either spontaneously or after exposure to various DNA-damaging agents [79••].

The DNA lesions resulting in double-strand breaks (DSBs) are among the most fatal, because they disrupt the continuity of the DNA template essential for DNA replication and transcription. A broken chromatid acquiring a new telomere leads to the loss of DNA distal to the break, resulting in LOH. DSBs could arise because of stalling of the replication fork during DNA synthesis; defective DNA repair; DNA damaging effects such as ionizing radiation; cleavage by specific enzymes such as V(D)J recombinase; or recurrent chromosomal breaks at susceptible DNA sequences caused by tandem repeat DNA instability at microsatellite (CCG)_n, AT-rich minisatellites, other minisatellites, or defined or unique signal sequences [80–84]. DSBs are highly recombinogenic and represent a major threat to the integrity of the genome. Although most DSBs are rejoined through repair pathways known as nonhomologous end joining (NHEJ) or homologous end joining (HEJ) pathways, they could also initiate homologous or non homologous strand invasion and recombination and repair [78,81,85,86••,87]. Mutations in the genes involved in these pathways display dramatic genomic instability ranging from chromosomal fragmentation to nonreciprocal translocations [88••,89,90••]. Homologous and nonhomologous recombination and repair, HEJ, and NHEJ involving homologous chromosomes and nonhomologous chromosomes would lead to partial chromosome homozygosity resulting in LOH (Fig. 2). The NHEJ and HEJ pathways would create translocations and isochromosomes, which could be detected cytogenetically using chromosome painting methods [19••].

Telomeres are specialized nucleoprotein complexes that serve to protect the ends of linear chromosomes from

recombination, fusion, and recognition as damaged DNA. Lack of functional telomeres in rapidly dividing cancer cells could occur because of defects in or low levels of telomerase or telomere binding proteins such as TRF1 and TRF2 and cause chromosome end-to-end (telomeric) fusion. The fusion-bridge-breakage process of chromosomes fused at their telomeres during chromosomal disjunction could initiate a wide array of chromosomal aberrations caused by the generation of DSBs, which could, in turn, cause a chain of events such as DNA strand invasions and chromosomal fusions [91•,92,93••]. If telomere attrition caused by loss of capping activity and severe genomic instability continues in cancer cells, it may lead to crisis and cell death [91•,93••]. Therefore, in most human cancers, telomerase usually becomes activated at the time of transition to advanced invasive cancer, enabling stable inheritance of these genetic alterations to the progeny cells [91•,92,93••]. The telomeric end-to-end fusions resulting in metacentric chromosomes and translocations involving portions of chromosomes also leads to partial chromosome homozygosity [19••] (Fig. 2).

Despite LOH analysis indicating loss of one of the chromosomes of the homologous pair caused by reduction to homozygosity based on all the markers analyzed, cytogenetic analysis by chromosome painting has revealed the existence of multiple copies of the chromosome being investigated [19••]. These results are consistent with defects in chromosome segregator genes coupled with duplication of the remaining chromosome, leading to whole chromosome homozygosity resulting in LOH (Fig. 2). The defects in chromosome segregator genes that are required for faithful disjunction of sister chromatids to the daughter cells could comprise genes that (1) participate in forming the centromere structure, (2) associate with the centromere during the metaphase to anaphase transition, (3) mediate the sister chromatid cohesion, and (4) play a role in the anaphase-promoting complex, including the proper assembly of the bipolar spindle apparatus, to ensure faithful segregation of genetic information [70,71,72••,73,74•,94,95,96••,97,98,99••]. However, study of these genes to determine the nodal targets most frequently affected in cancers is still in its infancy. Although Cahill *et al.* [100••] demonstrated inactivation of *hBUB1* and *hBUBR1* genes in colorectal cancers exhibiting chromosomal instability, subsequent progress has been slow, perhaps because of the large number of target genes that could become inactivated or the epigenetic mechanisms of inactivation of these genes [101,102,103••].

Conclusions

Despite an enormous amount of genomic sequence and contig data becoming available from the human genome project, the major challenge is to find the disease-causing genes that will make an impact on overall health care and

management. The exploitation of LOH analyses using traditional and high throughput methods such as bacterial artificial chromosome microarrays to define MLRs provides a distinct advantage to cancer geneticists in discovering target TSGs in this era of genomics. The LOH studies have not only provided a view of the genetic abnormalities of complex diseases such as cancer but also stimulated the drive to obtain a better understanding of the various cellular processes such as DNA repair, replication, recombination, telomere maintenance, and cell division by providing excellent, highly discernible visual examples resulting from their defects.

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APPENDIX II-Resume

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EDUCATION

University of Jaffna, Thirunelvely, Sri Lanka.	B.Sc. 1982	Biology
Bowling Green State University, Bowling Green, OH	M.S. 1986	Biology-Microbiology
The Johns Hopkins University, Baltimore, MD	Ph.D. 1992	Biochemistry
The Johns Hopkins Oncology Center, Baltimore, MD	1991 – 1995	PDF-Molecular Genetics/ Oncology

Graduate and Post-doctoral Advisors:

Doctoral Thesis Advisor: Dr. Lawrence Grossman, University Distinguished Service Professor of Biochemistry and Molecular Biology, The Johns Hopkins University, Baltimore, Maryland.

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PROFESSIONAL EXPERIENCE

1981 - 1982 Assistant Lecturer, Faculty of Science, University of Jaffna, Sri Lanka
1982 - 1984 Assistant Lecturer, Faculty of Science, Eastern University, Sri Lanka
1995 - 1998 Research Associate, The Johns Hopkins University School of Medicine
1998 - Assistant Professor of Medicine, Boston University School of Medicine
1999 - Assistant Professor of Pathology and Laboratory Medicine, Boston University School of Medicine
2002 - Assistant Professor of Genetic and Genomics, Boston University School of Medicine

AWARDS AND HONORS

1983 Overseas Research Students Award, CVCP of the Universities of the U. K.
1988-1991 Post Certified Student Scholarship, The Johns Hopkins University
1992-1995 Amgen Post-doctoral Fellowship
1995 Oncology Fellow Research day Poster Award
1995- Sterling Who's Who
1997- Who's Who in the East
1999 American Lung Association Research Award
1999 American Cancer Society Institutional Research Grant Award
2000-2002 The Dolphin Trust Investigator (New Investigator Award), The Medical Foundation, MA
2001 Burroughs Wellcome Fund New Investigator in Toxicological Sciences Award (Finalist)
2001-2005 Career Development Award, Department of Defense BCRP, U.S. Army MPMC

PROFESSIONAL ORGANIZATIONS

American Association for Cancer Research	-	Member
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EDITORIAL RESPONSIBILITIES

Editorial boards: Cancer Biology and Therapy, Journal of Molecular Biology and Biotechnology.

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SYNERGISTIC ACTIVITIES

- (i) Developed a double selection genetic assay to identify genes upstream of p53 function.
- (ii) Validated the double selection genetic assay for the identification of human p53 regulatory genes and isolated established regulators as well as previously unknown novel regulators such as hBUB1.
- (iii) One of the two major contributors to the discovery of five novel Smad genes (i.e., Smad1, Smad2, Smad3, Smad5, Smad6).
- (iv) Elucidated the molecular mechanism of loss of heterozygosity (LOH) in cancer with the use of molecular genetics and cytogenetics. These studies also confirmed that LOH is a reliable predictor for the genome localization of tumor suppressor genes.
- (v) Developed a novel method known as Targeted Expressed Gene Display (TEGD) for the discovery and/or analyses of a family of genes.
- (vi) Actively participates in training and educating the future generation of cancer researchers, educators and medical & public health practitioners by providing knowledge on the molecular basis of cancer and the application of genomic state of the art methodologies.